STUDIES ON VESICULAR-ARBUSCULAR MYCORRHIZA AND FUNGAL ROOT PATHOGENS OF WHITE CLOVER AND THEIR INTERACTION

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ABSTRACT

The role of a vesicular-arbuscular mycorrhizal fungus *Glomus fasciculatus* (Thaxter sensu Gerdemann) Gerdemann & Trappe in white clover (*Trifolium repens* L.) and its interaction with three fungal root pathogens *Codinaea fertilis*, *Fusarium avenaceum* (Fr.) Sacc. and *Thielaviopsis basicola* (Berk. & Br.) Ferraris was investigated.

The growth responses of white clover to applied phosphorus and *G. fasciculatus* infection are shown in two P-deficient soils applied with a range of phosphorus levels. Growth was greatly stimulated by *G. fasciculatus* in both soils at all phosphorus levels, but the magnitude of the mycorrhizal response decreased with increasing phosphorus levels.

Optical and transmission electron microscopes were first used to elucidate the mycorrhizal infection process and host response to infection. Some novel features including very thick-walled hyphae and intrahyphal hyphae within the host roots are recorded.

C. fertilis, *F. avenaceum* and *T. basicola* were shown to be pathogenic on white clover, the infection and colonization of the roots being followed microscopically. The pathogens showed variable effects on plant yield under various experimental conditions of defoliation, plant age and inocula type. *G. fasciculatus* was shown to confer protection to white clover by reducing the detrimental effect of the pathogens under some conditions.

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

1.1.1 Mycorrhizae

In the majority of plants growing under natural conditions, the organs through which they absorb water and nutrients consists not only of root tissue alone but root and fungus tissue (Gerdemann, 1968). These symbiotic associations between plant roots and highly specialized root-infecting fungi are known as mycorrhizae (fungus-roots). They have frequently been reported responsible for enhanced growth of plants or their survival in soils of low fertility.

Traditionally, mycorrhizae have been classified chiefly on the basis of structure into two major groups: ectotrophic and endotropic mycorrhizae, with the recognition that an intermediary type, the ectendotrophic mycorrhiza, exists also (Mikola, 1965).

The characteristic feature of ectotrophic mycorrhiza is that the fungal hyphae do not penetrate cells of the root but form a network (Hartig net) in the intercellular spaces and a complete sheath of tissue over the infected rootlets. In endotrophic mycorrhiza the hyphae penetrate cells of the root cortex but do not form a sheath over the rootlets. Connection with the soil is by individual hyphae or hyphal strands (Harley, 1969). Ectendotrophic mycorrhizal fungi produce both

Hartig net and intracellular hyphae in the cortex but the sheath over the rootlets may or may not be present (Laiho, 1965). In many recent publications (Harley, 1969, 1971; Ruehle & Marx, 1979; Smith, 1974, 1980; Malloch *et al.*, 1980) ectendotrophic type associated with roots of normally ectotropic mycorrhizal forest trees has either not been included or given little attention. This is due largely to the lack of information on the species of fungi involved in the association and their significance to tree growth (Marx & Krupa, 1978). Thus far, only limited research and observations have been made on this association (see Mikola, 1965; Laiho, 1965; Wilcox, 1971). Ectendotrophic mycorrhizae appear to have a limited distribution in forest soils and to be of lesser ecological importance than the other mycorrhizal types (Marx & Krupa, 1978). The association is considered by some to be a modification of the ectomycorrhizal type since it develops on some ectotrophic tree species only under certain conditions (Gerdemann, 1971).

The fungi which form mycorrhizal associations are now known to belong to a very diverse assemblage. They vary in taxonomic position, physiology and ecology. The mycorrhizae that they form also appear to show considerable diversity of structure and function (Smith, 1974). Over a number of years, various workers had endeavoured to improve the classification and nomenclature of mycorrhizae, taking into account physiology as well as structure of the associations.

Peyronel *et al.* (1969) proposed the substitution of the terms ectomycorrhiza and endomycorrhiza for ectotrophic and

endotrophic mycorriza, respectively. Lewis (1973) proposed substituting the term "sheathing mycorrhiza" for ectotrophic mycorrhiza, and three groups: vesicular-arbuscular, ericaceous and orchidaceous mycorrhizae for the associations originally included in endotrophic mycorrhizae. Those mycorrhizae within the endotrophic group, where insufficient information is available for generalizations to be made, are grouped by Lewis as "miscellaneous". Currently, Smith (1980) in her review, considered mycorrhizae in five main groups namely, ectotrophic, vesicular-arbuscular, orchidaceous, ericoid and arbutoid mycorrhizae.

Of the different kinds of mycorrhizae, vesicular-arbuscular (or VA) mycorrhiza is by far the most common. It occurs on an extremely diverse group of plants, from the Bryophytes, where the structure formed is called a mycothallus, to the Pteridophytes, Gymnosperms and Angiosperms (Gerdemann, 1968). It has been observed in 200 families and in more than 1000 genera (Malloch *et al.*, 1980). It is worldwide in distribution and occurs on plants from the Arctic to the Tropics and is present in most habitats (Gerdemann, 1968). It is this mycorrhiza which is the main concern of this thesis.

Ectotrophic mycorrhizae (or ectomycorrhizae) occur mainly on the important tree species around the world. They are found in all members of the Gymnosperm family Pinaceae (e.g. pine, spruce, fir and larch) as well as certain Angiosperms (e.g. willow, poplar, aspen, hickory, pecan, oak, birch, beech and eucalypt.) (Marx, 1980).

Orchidaceous mycorrhizae are found in the Orchidaceae; Ericoid mycorrhiza in the Ericaceae, Empetraceae and Epacridaceae (Malloch *et al*, 1980). Arbutoid mycorrhizae are found in the members of Ericaceous tribe Arbutaceae and sub-families Pyroloideae and Monotropoideae. It is interesting to note that arbutoid mycorrhiza has ectendotrophic features, with the production of intracellular hyphae as well as a sheath over infected rootlets but no Hartig net formation (Smith, 1980).

A great deal of research had been carried out on various aspects of mycorrhizal associations within the last thirty years. Reviews of literature on ectomycorrhizal associations are given by Marks and Kozlowski (1973), Marx and Krupa (1978); those on endomycorrhiza or vesicular-arbuscular mycorrhiza by Gerdemann (1968, 1975), Hayman (1978), Mosse (1963 , 1973a), Nicolson (1967) and Sanders *et al*. (1975) and finally those on mycorrhizae in general by Bowen (1979), Harley (1969), Malloch *et al*. (1980) and Smith (1974, 1980). In this chapter, only the pertinent literature central to the main themes of this thesis are reviewed.

1.1.2 Vesicular-arbuscular mycorrhizae

As with other mycorrhizal associations, there has been considerable controversy in the past concerning the identity of the endophytic fungi forming vesicular-arbuscular (VA) mycorrhizae. Many of the earlier difficulties were caused by the fact that these fungi appear to be obligate root-inhabiting fungi which still have not been satisfactorily grown in pure culture (Smith, 1974).

The lack of regular septa in their hyphae caused early workers to classify them as Phycomycetes and VA mycorrhizae have also been known as Phycomycetous mycorrhizae. This name, however, is no longer being used as the term Phycomycetes is now considered taxonomically defunct (Hayman, 1978). It was the observation that mycorrhizal roots were connected by hyphae to *Endogone* sporocarps which led Peyronel (1923) to suggest that VA mycorrhizal fungi were *Endogone* species. Later, Mosse (1953, 1956) also demonstrated experimentally that sporocarps of *Endogone* species produce VA mycorrhizae.

Prior to 1953 *Endogone* species were believed to be quite rare (Gerdemann, 1971). With the introduction of various methods to recover fungal spores from soil *Endogone* spores were found to occur in abundance in many parts of the world. The methods commonly used include wet-sieving and decanting (Gerdemann 1955; Gerdemann & Nicolson, 1963), a combination of the above method and centifuging in a sucrose density gradient column (Ohms 1957; Ross & Harper, 1970), differential sedimentation on gelatine columns (Mosse & Jones, 1968) and more recently the flotation-adhesion technique for recovery of spores from small soil samples (Sutton & Barron, 1972).

Originally, most of the spore types associated with VA mycorrhizae have been placed in the genus *Endogone*, family Endogonaceae and order Mucorales. These included zygosporic, chlamydosporic and sporangial species even though there was little evidence that the three spore types were related (Gerdemann, 1971). Different species within the genus were distinguished chiefly by the morphology of their spores. Mosse and Bowen (1968) have attempted to give the different

spores descriptive names based on distinctive morphological features.

With the recovery of more spore types over the years, the genus *Endogone* grew into an assemblage of diverse species about which few generalizations could be made. Within the genus, species differed in origin of spores, spore morphology, type of germination, mycorrhizal relationships and habitat (Gerdemann & Trappe, 1975). Consequently, Gerdemann and Trappe (1974) decided to make a revision of the genus *Endogone* sensu lato by recognizing three genera that had previously been described - *Endogone*, *Glomus* and *Modicella* and by describing a new genus - *Gigaspora*.

According to the classification of Gerdemann & Trappe (1974), the genera of Endogonaceae include *Endogone*, *Gigaspora*, *Acaulospora*, *Glomus* (including *Rhizophagus*, *Sphaerocreas* and *Stigeosporium*), *Sclerocystis* (including *Xenomyces* and *Ackermannia*), *Glaziella* and *Modicella*. Of these genera, those known to form VA mycorrhizae are *Acaulospora*, *Glomus* and *Sclerocystis*, while *Gigaspora* (except for *G. Calospora* which is reported to produce vesicles within roots - Furlan & Fortin, 1973) forms arbuscular mycorrhizae and *Endogone* forms ectomycorrhiza. Thus far, *Glaziella* and *Modicella* have not been known to form mycorrhiza (Gerdemann & Trappe, 1975).

Since the revision of the Endogonaceae by Gerdemann and Trappe (1974) several new taxa have been formally or informally described (see Hall & Fish, 1979) and a comprehensive key to the Endogonaceae has been made by Hall & Fish (1979). In this key, they included those formally and informally described taxa as well as those which have yet to be formally described.

1.1.3 Morphology and structure of VA mycorrhiza

The general morphology and structure of VA mycorrhizae of various plant species had been illustrated from early studies using the optical microscopes. Illustrations and descriptions of the structure of infection and development of these VA mycorrhizae, obtained within the limits of the resolution of the optical microscope, had been given by Jones (1924), McLuckie and Burgess (1932), Butler (1939), Nicolson (1959), Mosse (1959, 1963), Harley (1969) and reviewed by Nicolson (1967), Gerdemann (1968, 1975) and Hayman (1978).

One common characteristic feature of VA mycorrhizae of different plant species is the presence of vesicles and arbuscules in the root cortex. Inter and intracellular hyphae are also present in the cortex and the infection inside the root is directly linked to an external mycelium which spreads and ramifies in the soil (Hayman, 1978). Variations in detailed infection patterns, however, occur and appear to be influenced by factors such as the host species, the nutritional status of the host and the species of the endophyte (Bevege & Bowen, 1975; Gerdemann, 1965; Hall, 1977; Mosse, 1973b).

Scannerini and Bellando (1967) made the first detailed ultrastructural studies of a VA mycorrhiza of *Ornithogalum umbellatum* using the transmission electron microscope (T.E.M.). Following this work, electron microscopic techniques, and in particular the T.E.M. has been employed by various workers in the study of different aspects of VA mycorrhiza-host interactions.

Most reports of ultrastructural studies of VA mycorrhizae have been on the structures of infection and development in different host plants (Bonfante-Fasolo & Scannerini, 1977; Cox & Sanders, 1974, Holley & Peterson, 1979; Kaspari, 1973; Kinden & Brown, 1975b, c, 1976; Scannerini, 1972; Scannerini & Bellando, 1968; Scannerini *et al.*, 1975; Sward, 1978) and the mechanism of nutrient transfer in VA mycorrhizae (Cox *et al.*, 1975; Cox and Tinker, 1976). More recently, X-ray and electron probe microanalysis, histochemical, cytochemical and enzymological methods have been incorporated in the ultrastructural studies of various aspects of VA mycorrhiza-host interactions (Gianinazzi *et al.*, 1979; Schoknecht & Hattingh, 1976; Strullu *et al.*, 1981a, b; White & Brown, 1979), including features of the host-arbuscule interfacial matrix (Bonfante-Fasolo *et al.*, 1981; Dexheimer *et al.*, 1979; Scannerini & Bonfante-Fasolo, 1979). Information on the detailed ultrastructural features of the walls of intercellular hyphae is, however, lacking since observations have been limited mostly to the walls of the arbuscular hyphae or the interfacial matrix with the host.

1.1.4 Functions of VA mycorrhizae

It is now widely recognized that infection of plants with VA mycorrhizal fungi can lead to plant growth increases in nutrient poor soils. Reports of experiments showing growth increases of a wide range of plant species when inoculated with different VA mycorrhizal fungi can be found in reviews by Gerdemann (1968) and Mosse (1973a).

Evidence available to date suggests that the main effect of VA mycorrhizae on plant growth is through an increased efficiency in the uptake of poorly mobile ions by plant roots, in particular phosphate ions (Bowen, 1980). This is achieved by growth of extramatrical hyphae into soil, extending the root system, their absorption of ions and transport back to the plant. Evidence for increased uptake of phosphate by VA mycorrhizal fungal hyphae in soil has been given by various workers, including Sanders and Tinker (1971, 1973) and Hattingh *et al.* (1973). In many of the growth experiments with VA mycorrhizal fungi, similar growth increases could be reproduced by improved supplies of phosphorus to the plant, indicating that the main mycorrhizal effect is one of increased phosphorus nutrition. Increase in uptake of other ions, however, has also been shown. This includes uptake of zinc, strontium, potassium, copper and sulphate (Gilmore, 1971; Gray and Gerdemann, 1973; Jackson *et al.*, 1973; Jensen, 1982; Lambert *et al.*, 1979; Powell, 1975; Timmer and Leyden, 1980).

Plant growth depression, rather than stimulation, has also been reported for VA mycorrhizae (Smith, 1980). These growth depressions are of two types: transient and persistent. Transient growth depressions, usually smaller in magnitude, occur early in ontogeny in mycorrhizal plants which subsequently exhibit positive growth responses. They have been observed by Baylis (1971), Cooper (1975), Smith and Daft (1977), on different hosts infected with different mycorrhizal fungi. Persistent growth depressions usually occurring under conditions of high soil or plant phosphorus levels have been reported by Baylis (1967), Crush (1973), Hall *et al.* (1977) and Mosse (1973b).

The possible mechanisms for these growth depressions include:

(1) direct pathogenic effects of mycorrhizal fungi (Baylis, 1971; Furlan and Fortin, 1973),

(2) phosphate toxicity due to increased absorption by the VA mycorrhizae (Mosse, 1973b),

(3) competition of mycorrhizal fungi with plant roots for soil phosphorus (Crush, 1973) and

(4) excessive assimilate drain by the mycorrhizal fungi under particular conditions (Crush, 1976).

There is increasing circumstantial evidence that effects not directly related to nutrient uptake can occur with VA mycorrhizae. Bowen (1982) commented that almost all of the work suggesting physiological or other effects is inadmissible because large, high nutrient status mycorrhizal plants have been compared with small, nutrient poor plants. He suggested that the correct approach to such a problem would be to compare fertilized non-mycorrhizal plants with matched unfertilized mycorrhizal plants of the same size or the same phosphate content. Levy and Krikun (1980), in investigating water resistance of mycorrhizal and non-mycorrhizal citrus plants used matched plants and found that leaf conductivity and photosynthesis recovered more quickly in mycorrhizal plants on rewatering after a drying period. They suggested hormone effects to be involved.

The growth of VA mycorrhizal hyphae into soil could lead to other beneficial effects for plants growing in adverse situations. Several effects have been suggested by Bowen (1980). Many have not been investigated, or investigated by workers without using matched mycorrhizal and non-

mycorrhizal plants and await further study. These include:-

(1) Improved water absorption, especially in sandy soils with low moisture for plants with low rooting intensities.

(2) Counteracting toxicity of factors such as high and low pH, high aluminium and salinity. In the example of aluminium toxicity, Bowen (1980) suggested that VA mycorrhizal fungi might reduce toxicity by acting as a sink for aluminium, or by compensating for root loss.

(3) Structural effects in poorly structured soils. Sutton and Sheppard (1976) inoculated sterilized dune sand with a *Glomus sp.* and found five times the weight of sand aggregates/kg in mycorrhizal pots compared to non-mycorrhizal pots using *Phaseolus vulgaris* plants of similar size, the sand grains being held together by fungal hyphae.

(4) Nutrient conservation. In forest and grasslands it is probable that mycorrhizal associations play an important part in the conservation of nutrients and in nutrient cycling. Fungal hyphae readily penetrate litter and decomposing organic matter and can spatially compete with other soil micro-organisms for organic and inorganic nutrients far more efficiently than can plant roots.

(5) Compensation and avoidance of disease. Roots lost by disease attack in a deficient soil will lead to reduced yield but active mycorrhizae formation and hyphae growth into soil can compensate for such losses. For tree species, suberization or cutinization of newly formed roots confers a degree of disease protection but it also causes a very large decline in ion uptake by that part of the root. However, if the root has become mycorrhizal before suberization, it can maintain an efficient uptake function while protected from disease by the suberization.

1.1.5 Mycorrhiza in relation to plant diseases

The role of mycorrhiza in plant diseases has only really received attention in the last decade, although field observations relating ectomycorrhizae to decrease in root diseases were made much earlier (e.g. Levisohn, 1954). Investigations by various workers have confirmed the protective role of ectomycorrhizae in various feeder root diseases (Marx, 1972).

Zak (1964) postulated several mechanisms by which ectomycorrhizae may afford protection to feeder roots of plants. He suggested that ectomycorrhizal fungi may:-

(1) utilize surplus carbohydrates in the root thereby reducing the amount of nutrients stimulatory to pathogens,

(2) provide a physical barrier, the fungal mantle, to penetration by the pathogens,

(3) secrete antibiotics inhibitory to pathogens and

(4) support, along with the root, a protective microbial rhizosphere population.

In addition, Marx (1969) suggested that inhibitors produced by symbiotically infected host cortical cells may also function as inhibitors to infection and spread of pathogens in ectomycorrhizal roots. Literature on the interactions of ectomycorrhizae with feeder root pathogens has been reviewed by Marx (1972; 1982).

The interaction between VA mycorrhizae and plant pathogens has also been studied in the last ten years. Reviews of literature on this aspect were given by Hussey and Roncadori (1982), Schenck (1981), Schenck and Kellam (1978) and Schonbeck (1979). Most of these studies have concentrated on relation-

ships between VA mycorrhizae and soil-borne fungal pathogens causing root rots and vascular wilts, and plant-parasitic rematodes. There are relatively few reports of interactions between VA mycorrhizae and pathogens attacking aerial parts of plants.

In studies on the effect of VA mycorrhizae on fungal diseases of plants, mycorrhizae have been shown to cause an increase, or decrease, or have no effect on disease severity. The outcome of the interaction depends on the combinations of the host plant, fungal symbiont and pathogen. An example of VA mycorrhizal fungus in increasing disease severity is shown in soybean plants by Ross (1972). The pathogen, *Phytophthora megasperma*, caused internal stem discoloration symptoms of *Phytophthora* root rot in 90% of mycorrhizal plants, but in less than 20% of the non-mycorrhizal plants. An example of decreasing disease severity of *Thielaviopsis basicola* on cotton plants was shown by Schonbeck and Dehne (1977) with the mycorrhizal fungus *Glomus mosseae*. Shoot weights of mycorrhizal plants were significantly greater than non-mycorrhizal plants but the root weight did not differ significantly. Ramirez (1974), however, found that the three mycorrhizal fungi of papaya he used had no effect on root infection caused by *Phytophthora palmivora*.

The mechanism responsible for the protective effects of VA mycorrhizae in plant diseases are poorly understood. Unlike ectomycorrhizae, VA mycorrhizae do not have a fungal mantle as an external mechanical barrier and are not known to produce any antibiotics. Several protective mechanisms are suggested by some workers. Schonbeck (1979) suggested that

an enhanced strengthening of the cell walls in mycorrhizal roots by increasing lignification and production of other polysaccharides may be responsible for the increased resistance of mycorrhizal plants to pathogenic attack. Becker (1976) attributed the greater resistance of mycorrhizal roots to penetration of *Pyrenochaeta terrestris* to a greater abundance and formation of more developed callosities or lignitubers at the penetration sites. For mycorrhizal tobacco roots, the strong inhibition of chlamydospore production in *T. basicola* was shown by Baltruschat and Schonbeck (1975) to be due to a higher arginine content in the roots. A higher chitinase activity induced in the mycorrhizal roots of various host plants as found by Dehne *et al.* (1978), has been suggested by Schonbeck (1979) to be a possible factor for restricting the growth of pathogens within host tissues. Davis and Menge (1980), however, attributed the tolerance of citrus to *Phytophthora parasitica* root rot to the ability of mycorrhizal roots to absorb more phosphorus and possibly other minerals than non-mycorrhizal roots.

1.1.6 White clover (*Trifolium repens* L.): Role in farming and problems in persistence

Trifolium repens L., commonly known as white clover, is endemic to all European countries, most countries around the Mediterranean Sea, including some North African countries and many areas of Western Asia (Brougham *et al.*, 1978). It has since been introduced into many other temperate regions of the world and become one of the most important grazing legumes in many of these countries. To date, it is also the most import-

ant legume grown in New Zealand for general as well as intensive grazing (Dr. D. Scott, pers. comm.).

Like other legumes, white clover helps provide fixed atmospheric nitrogen to the associated grasses in pasture, while the clover herbage itself forms a highly nutritive component of the sward (Smetham, 1973). Amongst the *Trifolium* species, white clover is most suitable for use in intensively grazed swards because of its stoloniferous habit. Its other attributes include a wide habitat tolerance as well as its excellent colonizing ability.

Under favourable growth conditions, constant populations of white clover can persist for many years in mixed permanent pastures. This is attributed to its capacity for vegetative propagation from rooting stolons and to the process of natural reseeding (Crowder & Craigmiles, 1960). A lack of persistence of white clover from permanent pastures has, however, been a problem in some parts of the world, including some areas in the North Island of New Zealand. This was found to be mainly due to rotting of roots by pathogenic fungi. In the North Island of New Zealand and North Carolina in the United States, the main causal pathogen of root rot was *Codinaea fertilis* Hughes & Kendricks (Campbell, 1980; Menzies 1973a). In other parts of the United States, a complex of fungal species, with *Fusarium* being predominant have been associated with root rots of white clover (Hanson, 1953; Kilpatrick & Dunn, 1961; Leath *et al.*, 1971; Moody *et al.*, 1967).

It is generally agreed that the root rot problem of white clover, as in other legumes, is a complex one. Environmental factors play a very important role in the development of root rot and its severity. Vigorously growing plants in the field may not initially exhibit symptoms but may succumb to it gradually, often after periods of exposure to stress conditions. It is believed to be a product of the interaction of various factors of the physical and biological enviornment (Leath et al., 1971). Much work has been concerned with aspects of the environment (physical and biological) and management which pre-dispose plants to debilitating root rots. A review of literature on this aspect of root rot has been given by Latch and Skipp (In press).

1.2 OBJECTIVES AND SCOPE OF THE INVESTIGATION

It has been shown by various workers that VA mycorrhizal fungi are widespread in New Zealand soils (Crush, 1975; Neill, 1944; Powell, 1977) and invariably infect the pasture grasses and legumes growing in them (Crush, 1975). Various fungal species have been consistently isolated from white clover roots obtained from different pastures in New Zealand (Skipp, pers comm., Thornton, 1965). Apart from the poor persistence caused by *C. fertilis* in the North Island (Menzies, 1973a), very little is known about the root rot situation of white clover in other parts of New Zealand and the factors involved. In view of the importance of white clover, the widespread occurrence of VA mycorrhizal fungi in New Zealand pastures and the consistent association of white clover roots with soil-borne fungi, one of the main objectives of this work was to investigate the role of a VA mycorrhizal fungus, if any, in the root rot syndrome of white clover caused by soil-borne fungal pathogens.

The VA mycorrhizal fungus used for the interaction studies was *Glomus fasciculatus* (Thaxter sensu Gerdemann) Gerd. & Trappe, (Rothamsted E_3). Microscope studies of the infection and development of *G. fasciculatus* in white clover roots and the host response to infection were first made to gain information for a better understanding of the interaction with the root pathogens in subsequent studies. The T.E.M. was used to follow the development of mycorrhizal structures and an extensive study made of hyphae within the roots.

Various workers have found that growth responses of plants to VA mycorrhizal infection vary depending on the type of soil used and the level of soil phosphorus (Mosse, 1973b). The growth responses of white clover to inoculation with *G. fasciculatus* in two soils applied with different phosphorus levels were investigated in two experiments. The percentage of roots infected by *G. fasciculatus* at different phosphorus levels in the two soils was also assessed.

Various soil-borne fungi isolated from white clover roots were tested for pathogenicity on white clover seedlings under glasshouse conditions. Three of these fungal species, *Codinaea fertilis*, *Fusarium avenaceum* (Fr.) Sacc. and *Thielaviopsis basicola* (Berk. & Br.) Ferraris were found to be pathogenic. The features of infection and development of these pathogens in white clover root tissues and the host responses to infection were studied using microscopic techniques.

Preliminaty studies on the interaction of *G. fasciculatus* and the three fungal root pathogens were initially conducted, followed by an investigation of the responses of white clover to infection by *G. fasciculatus* and the three pathogens with respect to various important factors including defoliation, plant age and type of inocula.

CHAPTER 2

MORPHOLOGY AND ULTRASTRUCTURE OF *GLOMUS FASCICULATUS* IN WHITE CLOVER ROOTS

2.1 INTRODUCTION

Early studies on the morphology and structure of VA mycorrhizae revealed that these fungi typically produce vesicles, arbuscules, inter- and intracellular hyphae in the root cortex of the host plant (Hayman, 1978). Various workers, however, considered the morphology of VA mycorrhizae to be variable and affected by factors such as the nutritional status of the host, the host species and the species of the fungus (Hall, 1977; Mosse, 1973b).

Gerdemann (1965), found that *Endogone fasciculata* Thaxter (*Glomus fasciculatus* (Thaxter sensu Gerdemann) Gerdemann & Trappe) formed a VA mycorrhiza on tuliptree (*Liriodendron tulipifera* L.) that was almost entirely intracellular. The same fungus had been found to produce VA mycorrhizae with both inter- and intracellular structures in maize (*Zea mays* L.) (Gerdemann, 1965) and *O. umbellatum* (Bonfante - Fasolo & Scannerini, 1977). Other examples of VA mycorrhizae producing different infection patterns in different plant species are *Endogone araucareae* (Bevege & Bowen, 1975), *Sclerocystis rubiformis* (Hall, 1977) and various *Glomus* species (Chilvers and Daft, 1981). Mosse (1973b) and Hall (1977) also found that morphologically distinct infections were formed by a single VA mycorrhizal fungus in plants grown in soils differing in available phosphorus.

More recently, Abbott and Robson (1979) carried out a quantitative study of the anatomy of a *Glomus* species in different host species and under different nutrient levels. They came to the conclusion that the anatomy of the VA mycorrhiza was particularly stable under the conditions of their experiments.

In view of the variation in the findings for these different VA mycorrhizae and host species, the morphology of the mycorrhizae for each endophyte-host combination should perhaps be determined for the differing conditions of experiments and not inferred from the results of other VA mycorrhizae or the same VA mycorrhizae of different host species. The morphology and structure of the VA mycorrhizae produced by *Glomus fasciculatus* infection of white clover has not previously been described in detail. The main aim of this investigation was to study the infection and development of this VA mycorrhizal fungus in white clover roots, using microscopic techniques, with a view to gaining information for a better understanding of the interaction with root rot pathogens in subsequent studies. Studies using the transmission electron microscope revealed some novel features associated with the intercellular hyphae of *G. fasciculatus* within the host roots. Cytochemical staining for polysaccharide was used with the aim of providing further information on the detailed ultrastructure of some of these features.

2.2 MATERIALS AND METHODS

2.2.1 Soil

A soil collected from Cass and belonging to the Tasman silt loam set (N.Z. Soil Bureau, 1968) was used.

The soil was air-dried, passed through a sieve of 5 mm^2 mesh to remove root fragments and coarse stones and mixed with washed quartz sand, 2:1 by volume. The soil mixture was then spread thinly and fumigated with methyl bromide ('Velsicol Fumigant-1' with 98% methyl bromide, 2% chloropicrin) at 454 g/m^3 soil, under a plastic cover for 48 h. Fumigated soil was left vented for at least two weeks before use.

Fumigated soil mixture was weighed into 1050 g aliquots in polyethylene bags, which were then placed each in a 15 cm diameter 'Squat pot'. Basal nutrients containing 252 mg K_2SO_4, 918 mg $MgSO_4.7H_2O$ and 0.9 mg $Na_2MoO_4.2H_2O$ in 30 ml solutions were added onto the surface of the soil and allowed to dry. Lime ($CaCO_3$) was added at 2.25 g per pot of soil. This was then mixed thoroughly first with a glass rod and then by shaking in the bag for 30 s. The soil was then emptied into each of the 'Squat pots' standing in a 15 cm petri dish.

2.2.2 Mycorrhizal inoculum

The inoculum of *Glomus fasciculatus* used in this and subsequent experiments was obtained from an original pot culture of *G. fasciculatus* (Thaxter sensu Gerdemann) Gerdemann & Trappe (Rothamsted E_3) kindly supplied by Dr. I. R. Hall (Invermay Agricultural Research Centre, Mosgiel, New Zealand).

Chlamydospores of *G. fasciculatus* were obtained from soil and root fragments of the pot culture by using the wet sieving and decanting technique of Gerdemann & Nicolson (1963). Chlamydospores, either in loose clusters or sporocarps, were picked up by a pair of fine forceps (No. 5) from petri dishes containing a suspension of soil fractions retained on sieves with 500 µm, 210 µm, 149 µm and 74 µm mesh. Soil organic matter and dead root fragments were removed from among the chlamydospore clusters, which were then rinsed three times with sterile distilled water.

To inoculate white clover seedlings, 50 chlamydospores were transferred by a pair of forceps onto the roots of each 2-day old seedling at transplanting. The seedlings were allowed to grow for at least 8 weeks before mycorrhizal roots were used as inoculum.

To prepare the mycorrhizal inoculum, roots were carefully washed free of soil with tap water and rinsed three times with distilled water. The third washing was filtered through two layers of Whatman No. 1 filter paper and used for mycorrhizal control pots, to provide a common soil microflora. Mycorrhizal roots were cut into 10 mm long segments, randomly mixed and weighed into 0.5 g fresh weight aliquots.

2.2.3 Host plants

'Grasslands Huia' white clover seeds were surface sterilized with 0.5% sodium hypochlorite solution, rinsed three times with sterile distilled water, and inoculated with rhizobia (10 ml 'Rhizocote' inoculant in 100 ml sterile distilled water). The seeds were then left to germinate on

2% water agar at 25°C for 48 hours.

Three germinated seedlings were placed 10 mm above a pad of 0.5 g mycorrhizal root inoculum in the centre of each pot of soil. Control plants were inoculated with 2 ml of double-filtered washing per pot. One ml of rhizobial suspension was pipetted onto the soil around seedling roots in each pot. Seedlings were thinned to one after two weeks. The pots were housed in a glasshouse in which the air temperature fluctuated between 10°C and 30°C and watered with distilled water. Basal nutrients were also added to the soil once every two months.

Five seedlings were harvested 2, 3, 4, 6, 8, 12 weeks and 6 months after growing in the above conditions.

2.2.4 Optical microscopy preparation

The roots of mycorrhizal treated and control plants were carefully washed free of soil and examined visually for differences in external root morphology. A representative sample of the roots was then cleared and stained with lactophenol trypan blue according to the method of Abbott (pers. comm.) outlined in Appendix 1. After removing the excess stain with lactophenol, roots were mounted on microscope slides and examined under an optical microscope.

For serial thin sections, 0.5 cm mycorrhizal root segments were fixed in 3% glutaraldehyde made up in 0.1 M sodium phosphate buffer at pH 6.8, dehydrated by a tertiary-butyl alcohol series, embedded in 'Paraplast' Plus (Appendix 2) and sectioned to 10-15 µm thick using a rotary microtome.

The sections were mounted onto slides and stained with safranin-fast green (Appendix 3) or periodic acid-Schiff's stain (Appendix 4).

2.2.5 Transmission electron microscopy preparation

Roots from 6 month-old plants were used for this study. Root segments (1-2 mm long) from various regions of carefully washed root systems were fixed in an equal part mixture of 3% glutaraldehyde and 3% acrolein (made up in 0.1 M sodium phosphate buffer at pH 6.8). After several rinses in buffer, including an overnight wash, the samples were postfixed for 2 h in 2% OsO_4 at $4^{\circ}C$ in the same buffer. The fixed specimens were then rinsed in buffer, dehydrated in a graded series of acetone, infiltrated and embedded either in Spurr's (1969) resin or 'Araldite' (Fineran & Bullock, 1972). Thin sections were cut on an LKB ultrotome II, using glass and diamond knives and examined in an Hitachi HS-75 transmission electron microscope. Sections were viewed either unstained or after post-staining with uranyl acetate followed by lead citrate. Cytochemical staining for the detection of polysaccharide material was done on sections mounted on Formvar coated gold grids using the periodic acid - thiocarbohydrazide - silver proteinate (PA - TCH - AgPr) method of Thiéry (1967) with appropriate controls (Courtoy & Simar, 1974).

2.3 RESULTS

2.3.1 Visual and optical microscopic observations

Two weeks after inoculation, roots of some seedlings were observed to be infected with *G. fasciculatus* when viewed under the microscope. There was no change in external root morphology at this or later stages of mycorrhizal development. Mycorrhizal root systems are, however, much larger due to growth stimulation of the entire plant. A yellow pigment began to appear on mycorrhizal roots three weeks after inoculation, with the colour becoming more intense and widespread as the infection develops.

G. fasciculatus first forms an appressorium on the root surface and produces infection hyphae which penetrate the epidermal cells below (Plate 2.1A). They may form intracellular coils in one or two epidermal cells and then proceed as intercellular hyphae which grow from the outer cortex to the central cortical region.

As the intercellular hyphae spread longitudinally to the root axis in the central and inner cortex, lateral branches penetrate cortex cells. By repeated dichotomous branching of the invading hypha, a cluster of fine filaments known as the arbuscule is formed (Plate 2.1A, B). Two to three clusters of such filaments may arise in the same cell from different penetrating hyphae (Plate 2.2A). When newly formed, arbuscules often almost fill the volume of the cells (Plate 2.1B; 2.2A). However, the finer branches quickly degenerate and collapse leaving a much smaller irregularly lobed mass (Plate 2.2B).

Other structures observed are vesicles which develop inter- or intracellularly. They are oval or lemon shaped swellings borne terminally or occasionally in an intercalary position to the intercellular hyphae (Plate 2.1B). Lateral angular projections, commonly present on intercellular hyphae of other VA mycorrhizae, are also observed (Plate 2.1B; 2.2A). Other commonly observed features of intercellular hyphae of *G. fasciculatus* of white clover include Y-junctions (Plate 2.1A), H-junctions (Plate 2.1B) and occasional septation of the hyphae (Plate 2.1B).

The distribution of various structures of *G. fasciculatus* in root tissues was revealed by examination of whole root segments and serial thin sections. Infection of the epidermis and outer cortex is usually scanty, with intracellular hyphal coils in one or two cells immediately adjacent to penetration sites. Intercellular hyphae, arbuscules and vesicles are predominantly in the central and inner cortical regions. Apical meristems, endodermis and vascular tissues are usually not infected. Fine rootlets of older seedlings are usually intensely infected while taproots and larger lateral roots show sporadic infection. Mycorrhizal structures are not observed in roots where secondary growth has resulted in loss of cortex cells.

Roots of plants harvested on the third or fourth week after inoculation contain predominantly arbuscules; those harvested after the fourth week contain arbuscules and vesicles in varying proportions. In some older root segments, only vesicles and intercellular hyphae are observed, most of the arbuscules having degenerated.

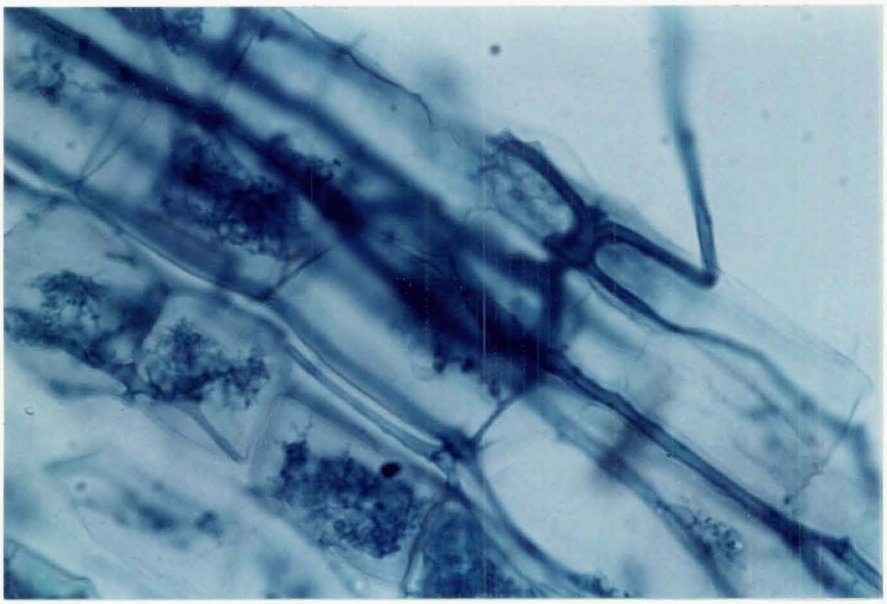
Plate 2.1 Infection features of *G. fasciculatus* in white clover roots. Whole root segments cleared with KOH and stained with lacto-phenol trypan blue.

A. Part of root showing the entry point of *G. fasciculatus*. Note the appressorium, the penetrating hyphae and the intracellular hyphal coil in the epidermal cells and arbuscules in the cortical cells. x1,300

B. Portion of a root intensely infected with *G. fasciculatus*. The central and inner cortical cells are filled with arbuscules and vesicles. Note the angular projections and the septa along some hyphae and the H-junctions produced. x1,300.

Plate 2.1

A.



B.

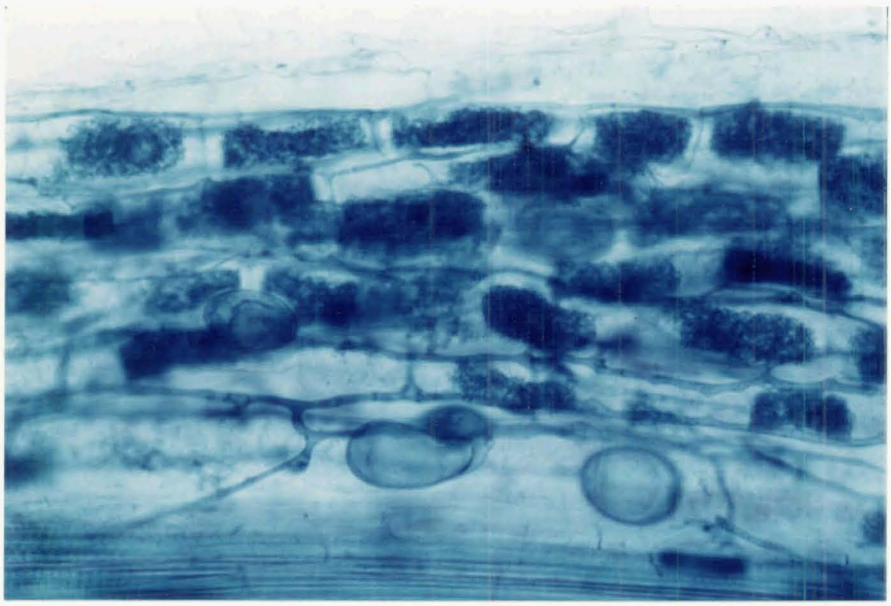


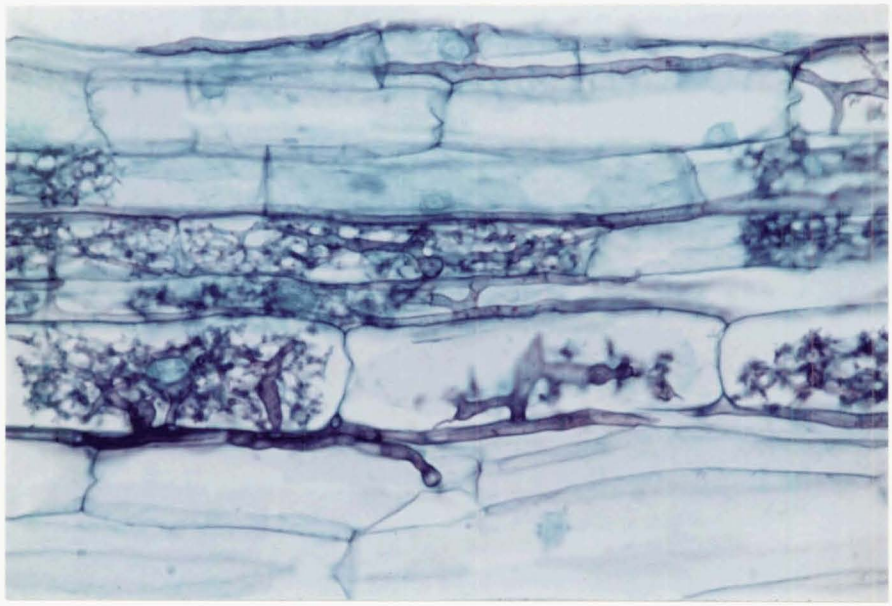
Plate 2.2 Arbuscules of *G. fasciculatus* at various stages of development and degeneration in the host cortex. Longitudinal sections of root, periodic acid and Schiff's stain.

A. Cortex cells showing dichotomous branching pattern and origin of arbuscular trunks from one or more lateral branches of intercellular hyphae. Enlargement of host nuclei occurred in cells containing arbuscules. x1,300.

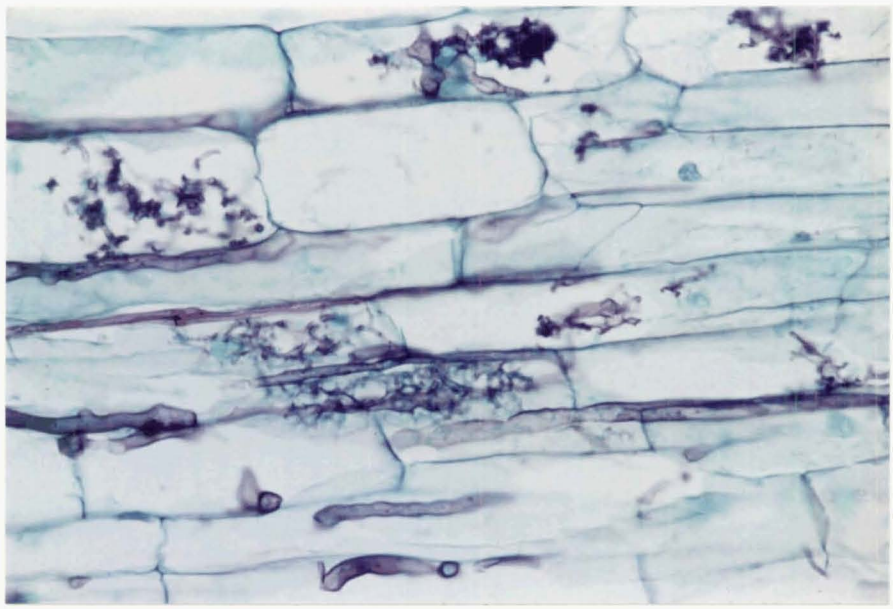
B. Cortex cells with arbuscules at advanced stages of degeneration. Note the irregularly lobed mass of collapsed arbuscular branches on the intact trunk hypha in one cell. x1,300.

Plate 2.2

A.



B.



In the younger roots of six month-old plants, infection features are similar to those described previously. A great proportion of roots, however, contain mainly coarse, intercellular hyphae with Y-junctions and many large oval or round vesicles (Plate 2.3A). Some of the coarse hyphae are connected to hyphae of smaller diameter, but separated from them by septa. The narrow hyphae are without visible contents and are collapsed at various points. Few arbuscules are observed in these old roots and those that are observed are at advanced stages of degeneration.

On some roots that had been carefully washed to remove adhering soil particles, an extensive hyphal network often remained on the root surface linked to hyphae within the roots. The major branches are darkly stained and non-septate. Many lateral branches, however, appeared less darkly stained and are septate (Plate 2.3B).

2.3.2 Transmission electron microscopic observations

2.3.2.1 Arbuscule development and degeneration

In the central and inner cortex, lateral branches from intercellular hyphae penetrate the cells to form arbuscular trunk hyphae. The arbuscular trunk is completely surrounded by the host plasmalemma but separated from it by an apposition layer in an electron-translucent space of varying widths (Plate 2.4A, B). This apposition layer is continuous from the inner host wall layer and identical with it in fibrillar texture (Plate 2.4A) and staining reactions when treated with uranyl acetate and lead citrate (Plate 2.4A,B), and the PA-TCH-AgPr method of Thiéry (1967) (Plate 2.7B).

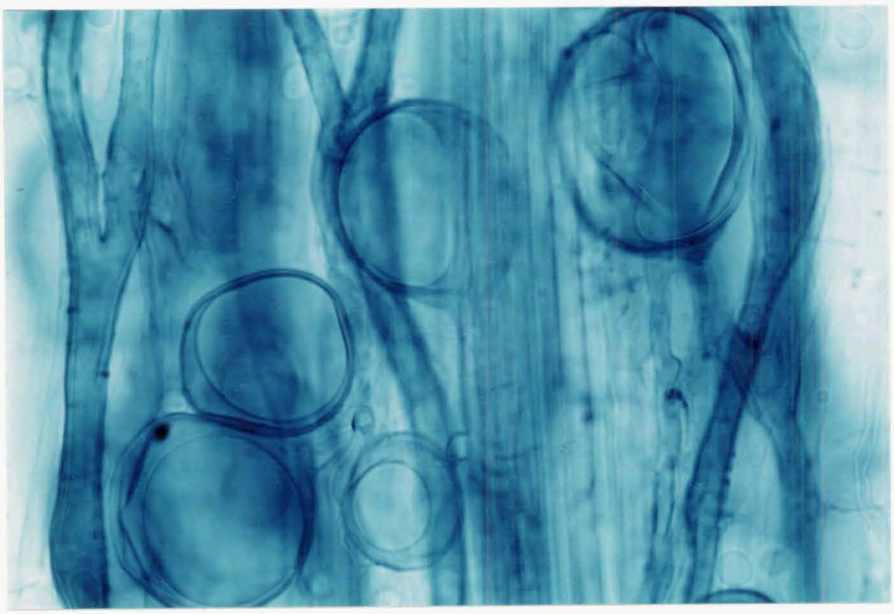
Plate 2.3 *G. fasciculatus* infection features in old white clover roots. Roots cleared with KOH and stained with lactophenol trypan blue.

A. Intercellular hyphae and vesicles of *G. fasciculatus* in the cortex of old clover roots. x1,300.

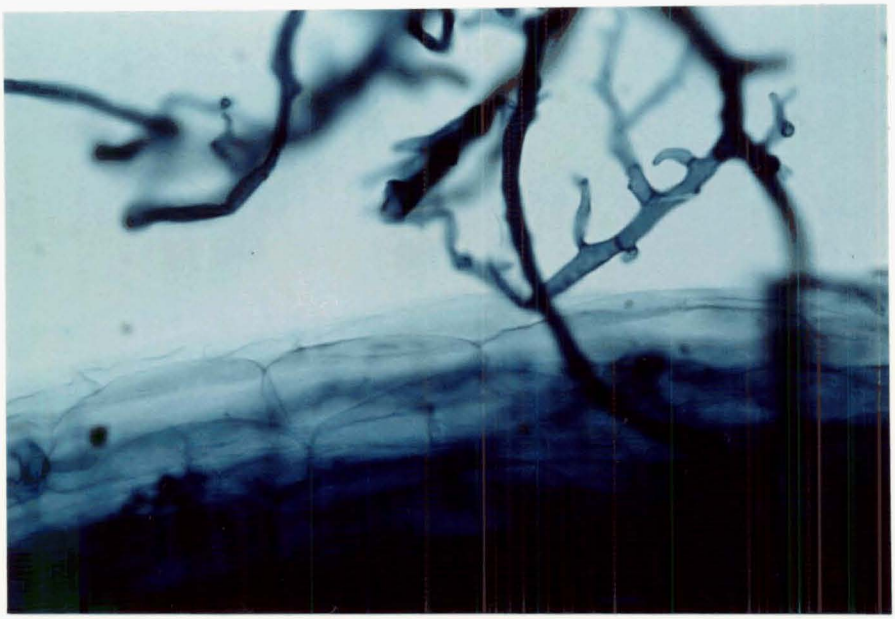
B. Part of a root segment showing the attachment of extra-matrical hyphae to structures within the root. The main branches are darkly stained and non-septate, the lateral branches stained lightly and are septate. x825.

Plate 2.3

A.



B.



Within the host cortex, the arbuscular trunks (diameters between 1.5 to 3.5 μm) branch dichotomously into progressively smaller branches. The smallest arbuscular branches measure between 0.25 to 0.28 μm in diameter. All the arbuscular branches are surrounded by the host plasmalemma but separated from it by an interspace containing an electron-dense matrix, which varies in quantity and appears either amorphous or fibrillar in texture (Plate 2.4C; 2.5A, B). Sections treated with PA-TCH-AgPr show that the quantity of this matrix around the branches is less than that around the arbuscular trunk (Plate 2.7B).

Host cells respond to intracellular development of arbuscules by an increase in the number of mitochondria, plastids and other organelles in the cytoplasm and an enlargement of the nuclei. The cytoplasm with its organelles occur mainly around the arbuscular branches (Plate 2.4C; 2.5B). It is separated from the walls of the arbuscule by the host plasmalemma and the interfacial matrix (Plate 2.4C; 2.5A, B). Abundant vacuoles with tonoplasts also occur among the arbuscular branches. Starch granules are usually not observed in cells containing arbuscules.

The arbuscular branches, when newly formed, contain a layer of darkly stained wall and cytoplasm consisting of ribosomes, nuclei, mitochondria and other organelles necessary for metabolic activities. The cytoplasm quickly degenerates and condenses. Vacuoles appear, and in the smaller branches vacuoles sometimes occupy the entire cross-section of the hyphae (Plate 2.4C; 2.5A, B). The walls of these vacuolated or empty branches eventually collapse.

Collapsed arbuscular branches frequently become separated from the functional part of the arbuscule by the formation of septa (Plate 2.5A, C). Degeneration of cytoplasm and collapse of the hyphal wall appears to progress from the smaller to the larger branches (Plate 2.4C; 2.5C; 2.6A).

Collapsed walls gradually become aggregated into compact masses. The host plasmalemma around each individual arbuscular branch eventually encloses the mass as a whole, with a loose network of disorganised fibrils present among the wall fragments (Plate 2.5C). Within the compact mass, collapsed fungal walls undergo fragmentation and distortion (Plate 2.5D).

In many cases, the arbuscular trunk hyphae remain intact in cells after the smaller branches have degenerated into the clump stage or disintegrated completely. These hyphae typically have variously thickened walls and cytoplasm containing large numbers of glycogen and lipid globules (Plate 2.4B; 2.6A, B, C). In extreme cases, the walls of the trunk hyphae are thickened to the extent of causing near occlusion of the hyphal lumen (Plate 2.6C). In host cells which remain alive on degeneration of the arbuscules, starch granules begin to reappear (Plate 2.7A). These cells are occasionally observed to be reinvaded by other soil fungi or by hyphae of *G. fasciculatus* producing new arbuscules.

Plate 2.4 Growth and development of arbuscules in the root cortex. Sections post-stained in uranyl acetate/lead citrate. Bar scale = 1.0 μm.

Abbreviations:

HC - host cortical cell; Hv - host vacuole;
Hm - host mitochondrion; Ps - plastid;
FW - wall of hypha; V - fungal vacuole;
L - lipid globule.

Host cell wall indicated by arrowheads.

A. Transverse section of a subtending intercellular hypha and longitudinal view of an arbuscular trunk hypha penetrating a cortex cell. The protoplast of the trunk hypha had degenerated. Note the apposition layer (arrows) between the host plasmalemma (curved arrow) and the walls of trunk hypha. x22,000.

B. Slightly oblique transverse section of a matured arbuscular trunk hypha in a cortical cell. The trunk hypha is completely surrounded by the host plasmalemma (curved arrow) but separated from it by an apposition of host wall material (arrow). x19,000

C. A matured arbuscule in a host cortex showing longitudinal and transverse sections of various arbuscular branches. Some small branches had collapsed (curved arrows). x18,000.

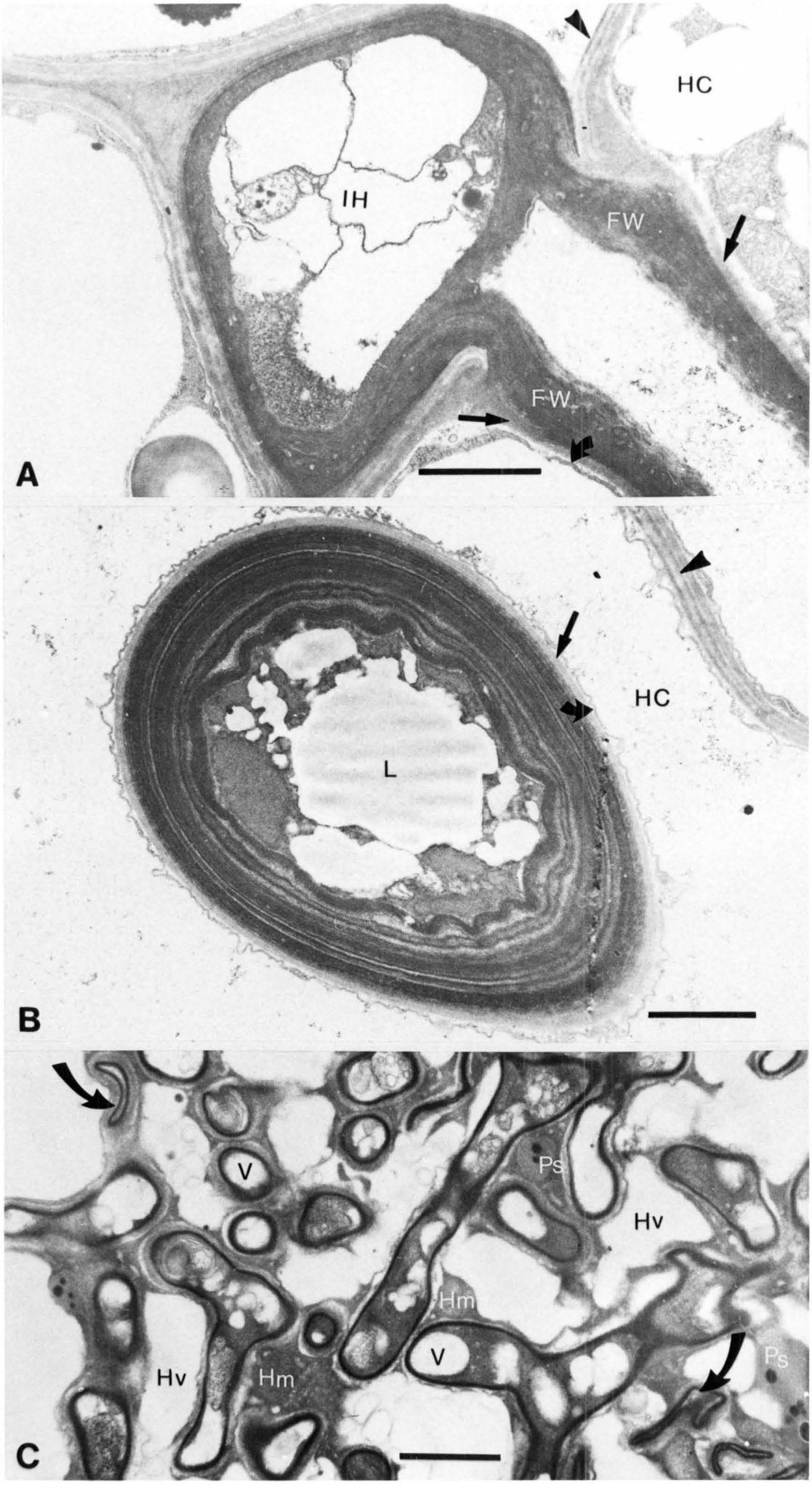

HC
IH
FW
FW
A
HC
L
B
V
Ps
Hv
Hm
V
Hv
Hm
Ps
C

Plate 2.5 Maturation and degeneration of arbuscules in the host cells. Sections post-stained in uranyl acetate/lead citrate. Bar scale = 0.5 µm for A; 1 µm for B, C, D.

Abbreviations:

Hn - host nucleus; Hv - host vacuole; Hm - host mitochondrion; IH - intercellular hypha; Ps - plastid.

A. Arbuscule at early stage of degeneration. Note the septum (open curved arrow) separating the collapsed from the intact portions of an arbuscular branch. x21,000.

B. Arbuscule branches in a host cortex containing cytoplasm with abundant mitochondria and plastids. Note the apposition material (asterisks) between the arbuscule walls and the host plasmalemma (curved arrow) and the host cytoplasm between the plasmalemma and the tonoplast (arrow). x21,000.

C. Collapsed walls of a major and smaller arbuscular branches. Note the septum (open curved arrow) in the major branch and the disorganized fibrils among the collapsed wall fragments. Curved and straight arrows indicate host plasmalemma and tonoplast respectively. x20,000.

D. A compact mass of collapsed and fragmented arbuscular walls surrounded by the host plasmalemma (curved arrow) and the tonoplast (arrow). x12,000.

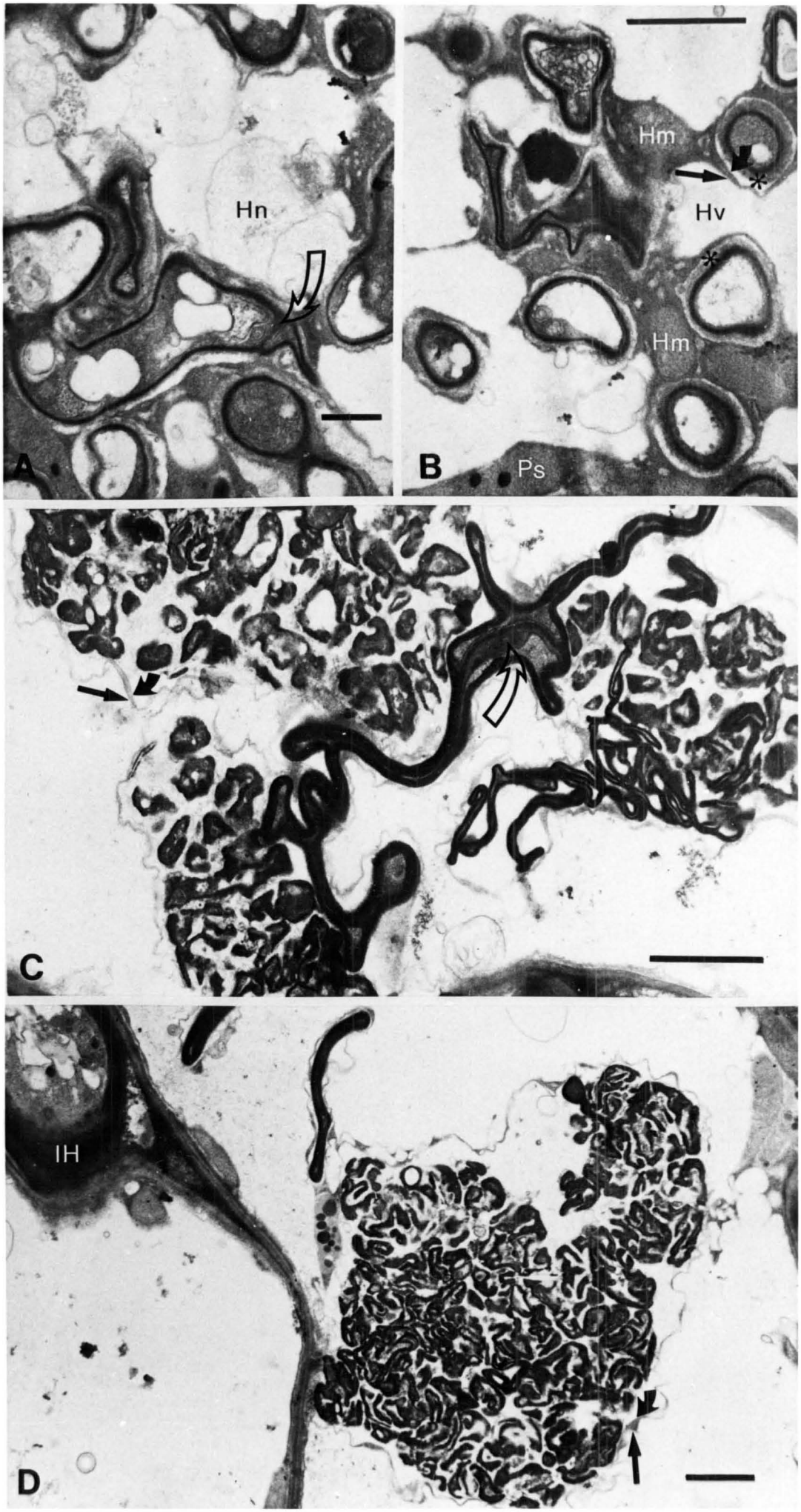

Hn
A
Hm
Hv
Hm
B
Ps
C
IH
D

Plate 2.6 Arbuscules at advanced stages of degeneration. Sections post-stained in uranyl acetate/lead citrate. Bar scale = 1.0 µm.

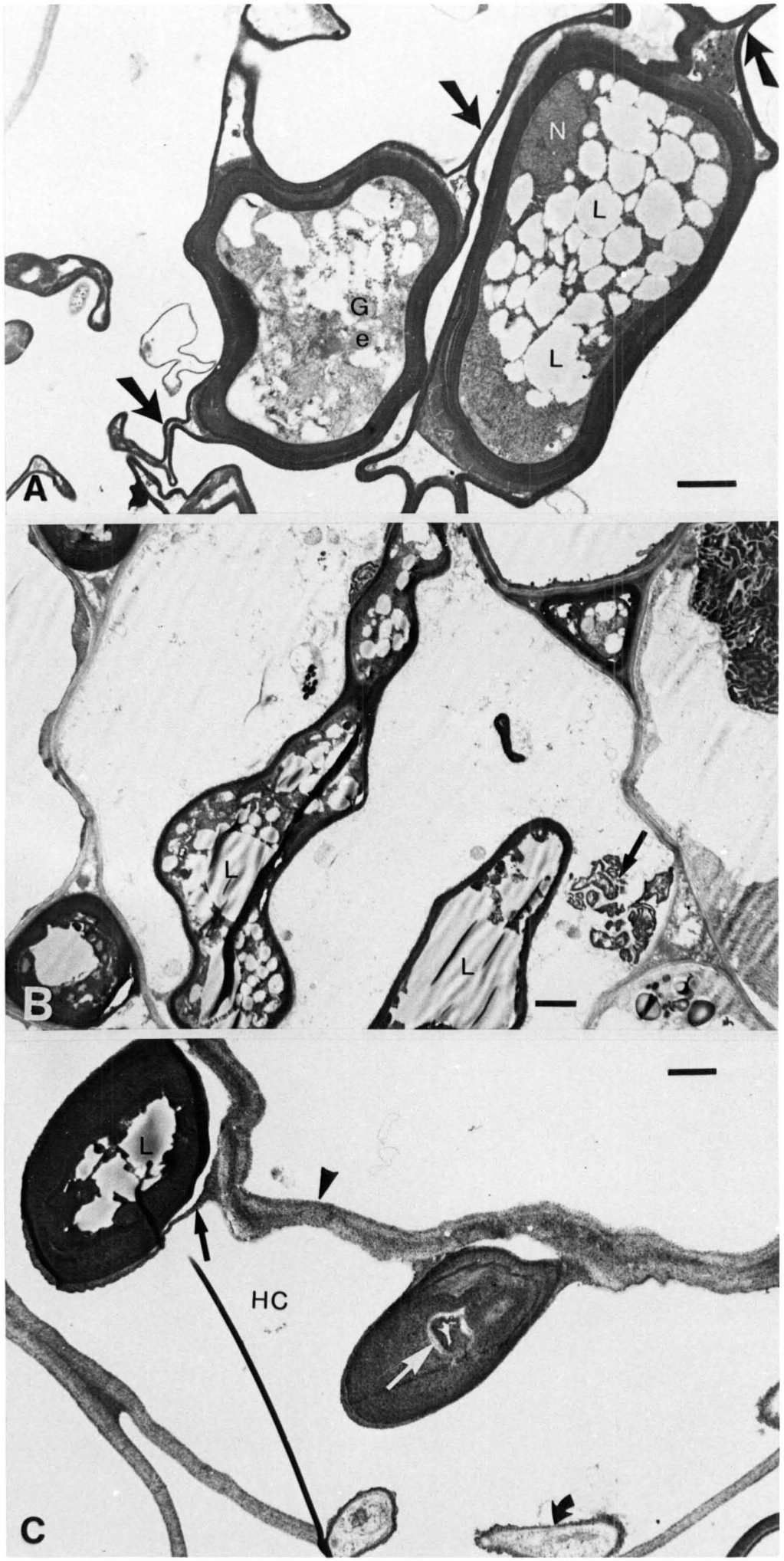
Abbreviations:

N - fungal nucleus; e - electron dense body; L - lipid globule; G - glycogen body.

A. Cortex cell with intact arbuscular trunk hyphae connected to collapsed walls of major branches (arrow) showing the progression of the degeneration process. x10,000.

B. Longitudinal section of two intact arbuscular trunk hyphae in cortex where the small arbuscular branches had collapsed (arrow). x7,000.

C. Oblique transverse section of a cortical cell with thick walled arbuscular trunk hyphae. The lumen of one trunk hypha (white arrow) is almost occluded by the thickened wall. Other hyphae (curved arrow), probably of a different fungus, have invaded the cell. Host wall and apposition layer around the arbuscular trunk indicated by arrow head and arrow respectively. x8,000.


N
L
G
e
L
A
L
L
B
L
HC
C

The walls of the arbuscules showed a positive reaction for polysaccharide material after PA-TCH-AgPr treatment (Plate 2.7B; 2.8). There is, however, a difference in the intensity of staining of the arbuscular trunk and the collapsed branches. The intact arbuscular trunk and the newly collapsed large branches give the strongest positive reaction, while the aggregated collapsed wall fragments of the small branches, the weakest reaction (Plate 2.8). The apposition layer around the arbuscular trunks shows a similar intensity of staining as the host cell wall to PA-TCH-AgPr treatment. It also appears thicker than the layer around the arbuscular branches (Plate 2.7B). Sections treated with uranyl acetate followed by lead citrate do not show a great difference in intensity of staining of the intact arbuscular trunk or collapsed walls of the major and smaller branches (Plate 2.5C, D; 2.7A).

2.3.2.2 Inter- and intracellular vesicles

Vesicles, produced both inter- and intracellularly, are a common feature observed in older roots of the host plants. Vesicles produced intercellularly cause a distention of the intercellular spaces and compression of adjacent cortex cells (Plate 2.9A). Intracellular vesicles are produced either in empty cortex cells or in cells with fragments of degenerated arbuscules (Plate 2.9B). At maturity, they may fill the entire volume of the cell, being surrounded on the outside by an apposition layer of host wall-like material and sometimes conform to the elongated shape of the cells in longitudinal view (Plate 2.9C).

Plate 2.7 Differential staining reactions of arbuscule walls. Section in A post-stained in uranyl acetate/lead citrate. Section in B treated with PA-TCH-AgPr. Bar scale = 1.0 μm.

Abbreviations:

S - starch granules; IH - intercellular hypha.

A. Portion of a host cortex containing a degenerated arbuscular clump with intact trunk hypha. A number of starch granules have reappeared in the host cytoplasm. The walls of the trunk hypha (arrow), collapsed major (open curved arrow) and smaller branches (curved arrow) show only slight differences in intensity of staining. x12,000.

B. A cortex cell containing arbuscule with walls showing positive reaction to PA-TCH-AgPr treatment. The apposition layer appears thicker around the trunk hypha (arrow) than the arbuscular branches (curved arrows) and it shows similar staining intensity to the host wall (arrowhead). x13,000.

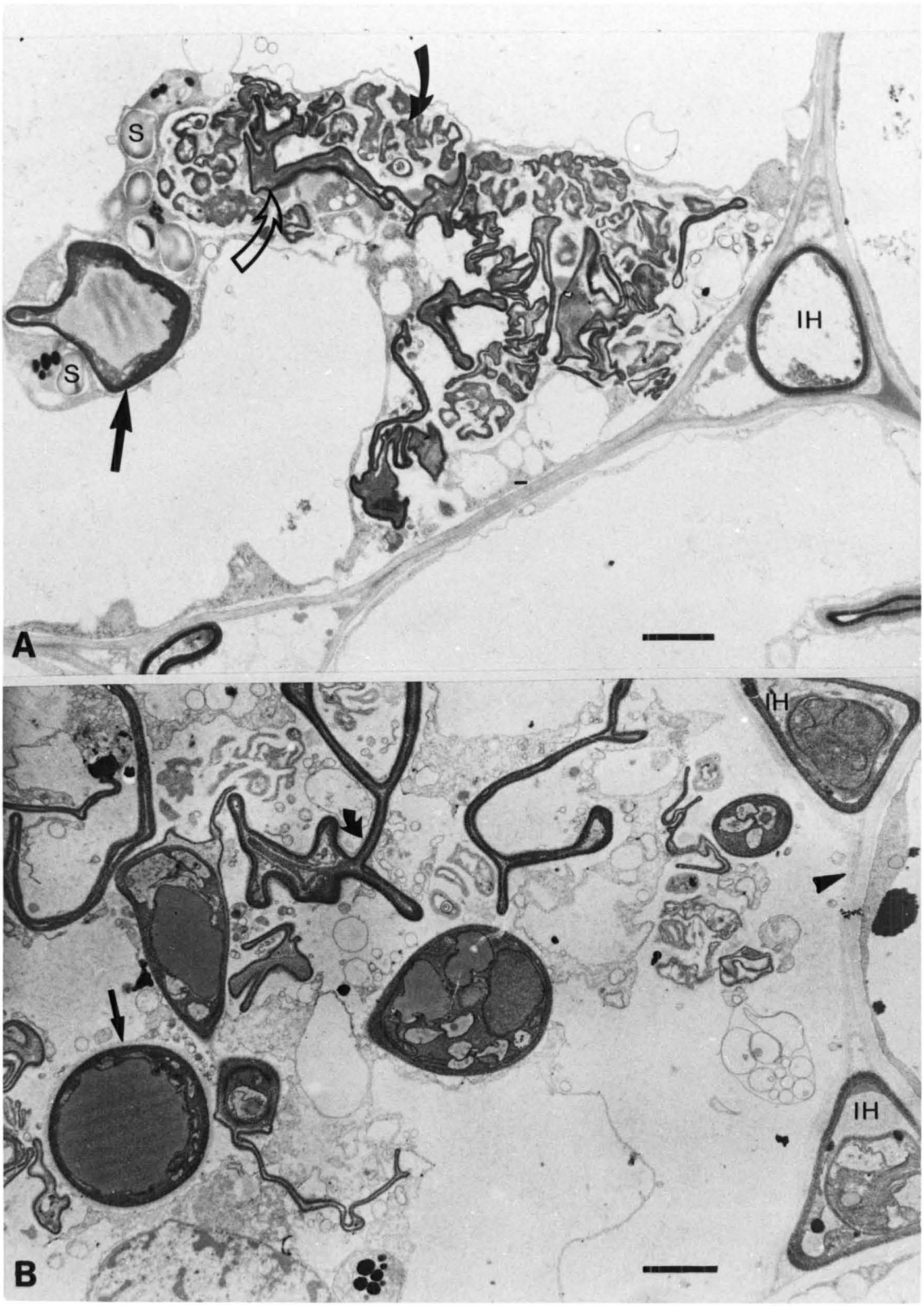
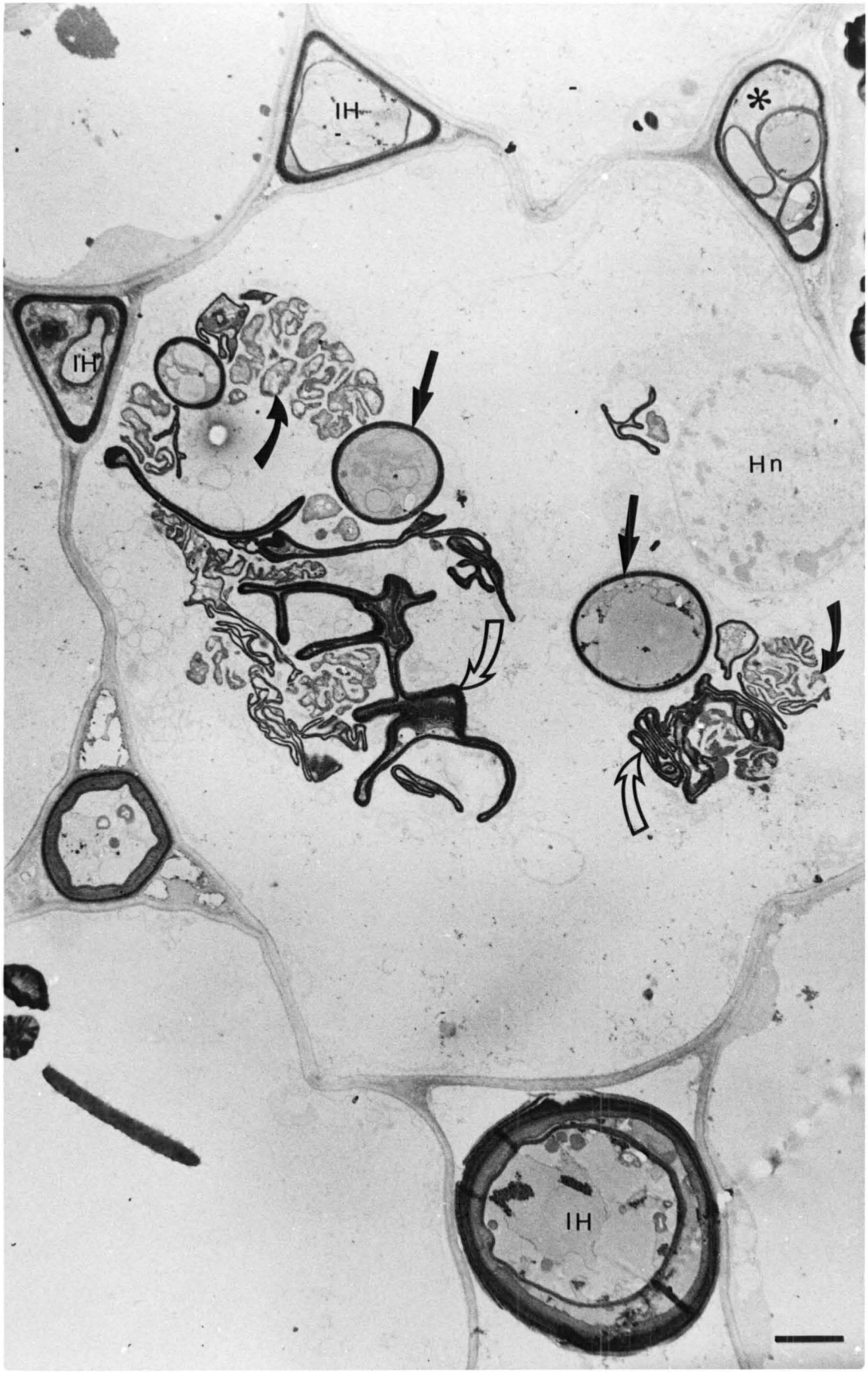

S
S
IH
A
IH
IH
B

Plate 2.8 Transverse section of the host cortex treated with PA-TCH-AgPr showing differential staining reactions of the arbuscular walls. The walls of arbuscular trunk (arrows) and collapsed major branches (open curved arrows) stain more darkly than the collapsed smaller branches (curved arrows). Intercellular hyphae around the cortex vary in sizes and wall thickness. Three of them show intrahyphal hyphae development (IH), one shows multiple infection (asterisk). x12,000. Bar scale = 1.0 μm

Abbreviation:

Hn - host nucleus.


IH
IH
*
Hn
IH

Ultrastructurally, no distinguishable differences were observed between the inter- and intracellular vesicles. In developing vesicles, large numbers of nuclei, mitochondria, ribosomes, small vacuoles and electron dense bodies are present in the cytoplasm (Plate 2.10A, B, C, D). As the vesicles mature, the cytoplasm condenses and the organelles become indistinct. Eventually the vesicles become filled with large lipid globules formed by coalescence of the smaller globules, and become surrounded by thin bands of condensed cytoplasm (Plate 2.9C; 2.10E). The walls of mature inter- and intracellular vesicles are thick (1.5 - 1.8 µm) and appear multilaminated (Plate 2.9C; 2.10E), with alternating dark and light staining bands.

2.3.2.3 Intercellular hyphae

Hyphae of *G. fasciculatus* occur abundantly in the intercellular spaces of the host cortex. They vary in their ultrastructural features. Thin-walled hyphae containing metabolically active protoplasts are observed (Plate 2.11C). Most hyphae, however, in the older portions of the root have variously thickened walls. The protoplasts are either filled with lipid and vacuoles (Plate 2.11A, B) or condensed and contracted (Plate 2.13 D, E). Hyphae, devoid of contents, also occur occasionally (Plate 2.11B).

The intercellular hyphae vary in size, but usually are less than 6 µm in diameter. The very young hyphae, with average diameter slightly less than 2.0 µm, typically have thin walls and dense cytoplasm containing nuclei, ribosomes, mitochondria and occasionally glycogen bodies but few, if any, vacuoles (Plate 2.12A, B). As the hyphae

Plate 2.9 Inter- and intracellular vesicles of *G. fasciculatus* in the host cortex. Sections post-stained in uranyl acetate/lead citrate. Bar scale = 1.0 μm.

Abbreviations:

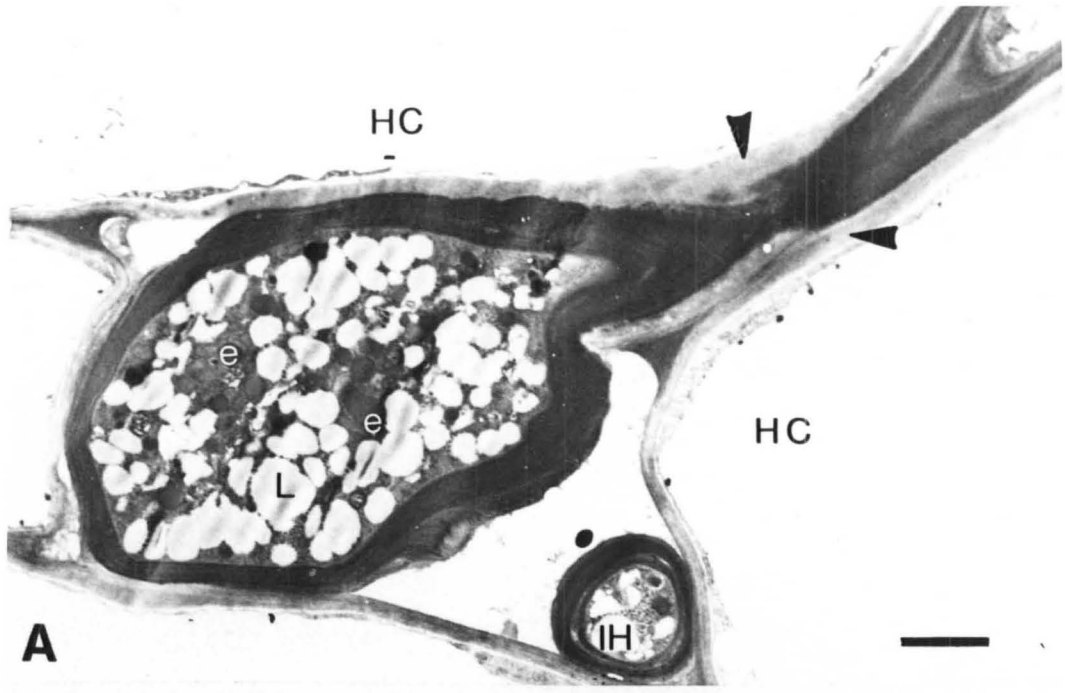
HC - host cortical cell; IH - intercellular hypha; L - lipid bodies; e - electron dense body; VW - vesicle wall.

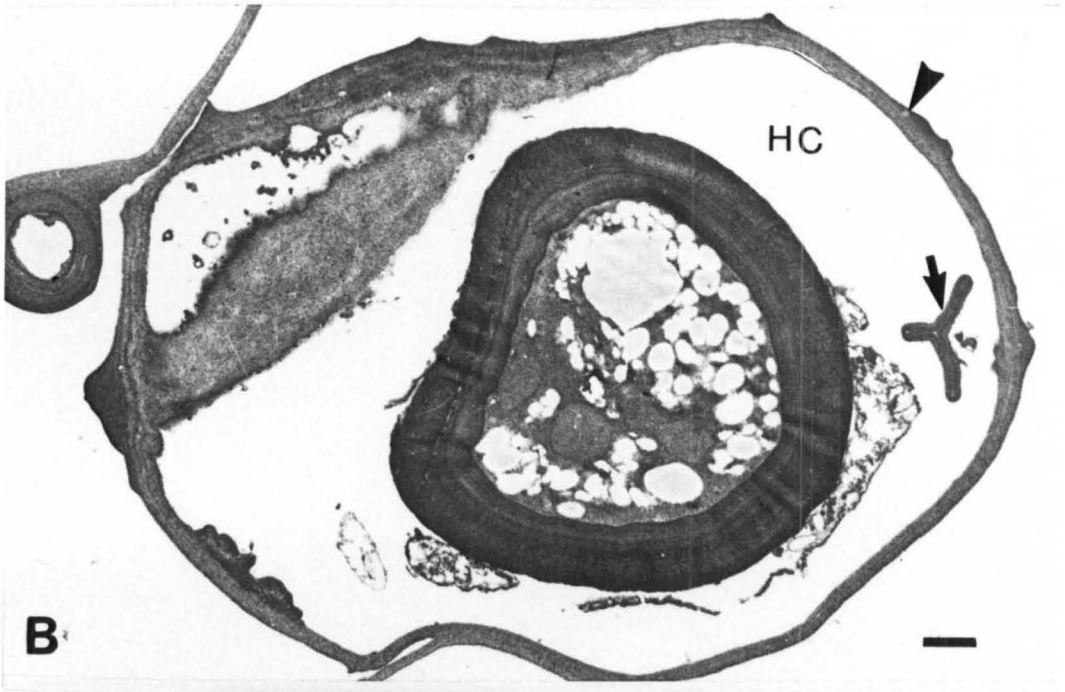
Host wall indicated by arrowheads.

A. A young vesicle developing in the intercellular spaces of cortical cells. Note the abundance of small lipid globules and electron dense bodies in the cytoplasm. x10,000.

B. A vesicle produced in an empty cortical cell containing only fragments of degenerated arbuscule (arrow). x6,000.

C. A matured intracellular vesicle occupying the entire volume of the cortex cell. Note the apposition layer (arrow) separating the host wall from the thick multilaminated vesicle wall. The cytoplasm of the vesicle is condensed and contains large lipid bodies. x10,000.


HC
e
e
L
HC
IH
A


HC
B

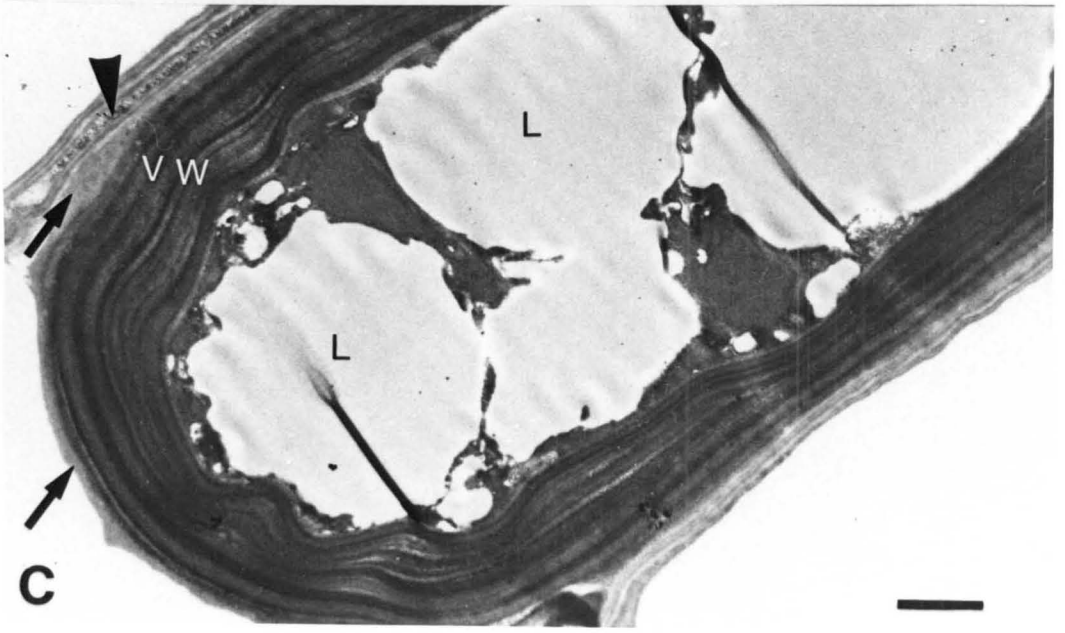

L
V W
L
C

Plate 2.10 Detail ultrastructure of developing and matured vesicles. Sections post-stained in uranyl acetate/lead citrate. Bar scale = 1.0 μm.

Abbreviations:

N - nucleus; R - ribosomes; M - mitochondrion; G - glycogen bodies; e - electron dense body; L - lipid globule; VW - wall of vesicle.

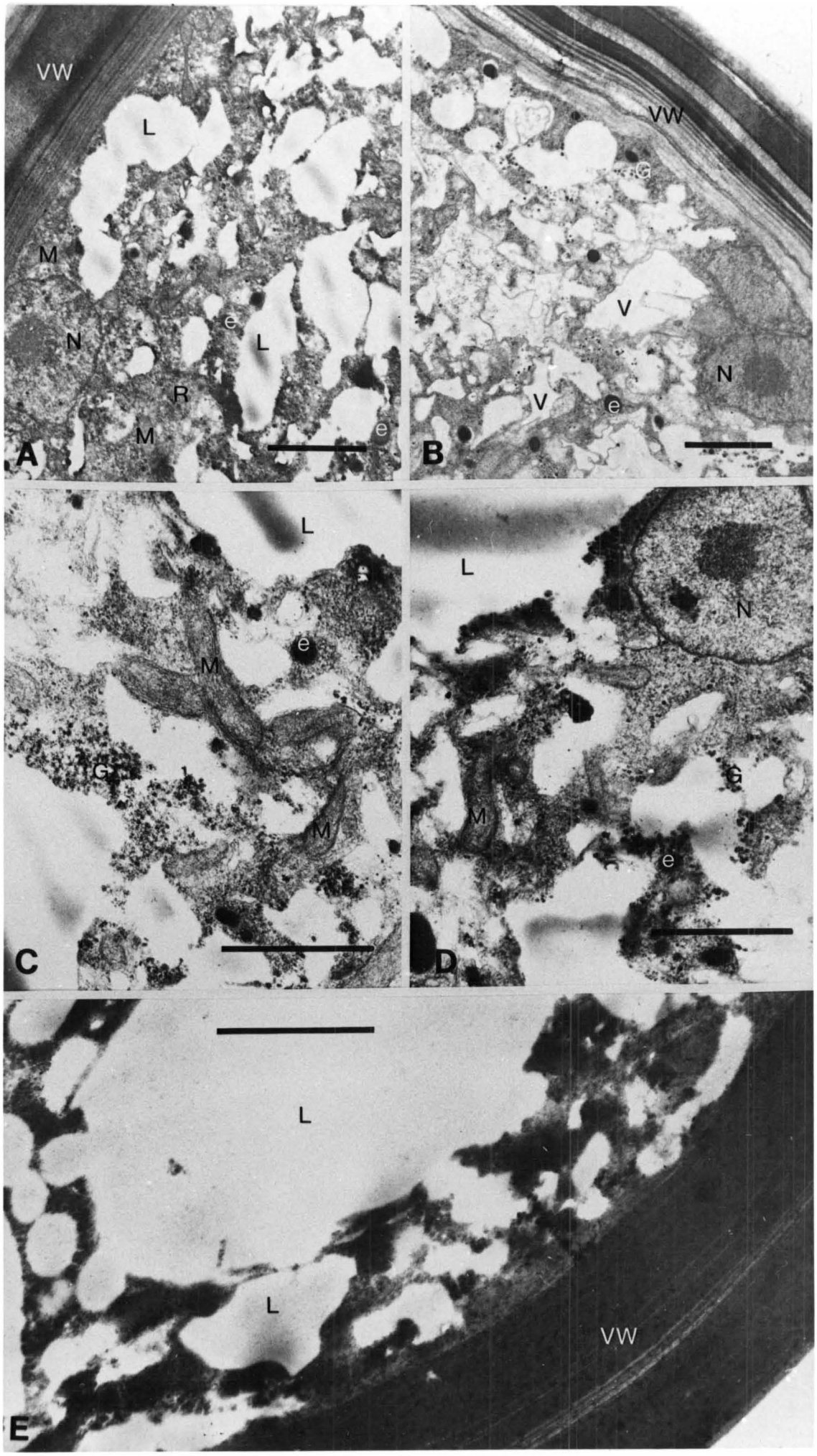
A. Transverse section of a portion of developing intercellular vesicle. The cytoplasm, enclosed by a darkly stained multilaminated wall contains nuclei, ribosomes, mitochondria, glycogen and electron dense bodies, and lipid globules. Note the coalescing of smaller lipid globules into larger ones. x18,000.

B. Transverse section of a portion of intercellular vesicle at about the same stage of maturity as the vesicle shown in A. Note the large number of small vacuoles surrounded each by a tonoplast. The thick wall shows alternating dark and lighter staining bands. x16,000.

C. Transverse section of a developing intracellular vesicle showing abundant mitochondria, glycogen and electron dense bodies and lipid globules in the cytoplasm. x28,000

D. Transverse section of similar vesicle as shown in C, showing a nucleus surrounded by its double membrane, in addition to the other cytoplasmic organelles. x26,000.

E. Portion of a matured intercellular vesicle showing darkly stained thick multilaminated wall and condensed cytoplasm with indistinct organelles and large lipid bodies. x29,000.


VW
L
M
N
e
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R
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A
VW
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B
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M
C
L
N
G
M
e
D
L
L
VW
E

Plate 2.11 General appearance of *G. fasciculatus* hyphae in the intercellular spaces of host cortex. Sections post-stained in uranyl acetate/lead citrate. Bar scale = 1.0 µm.

Abbreviations:

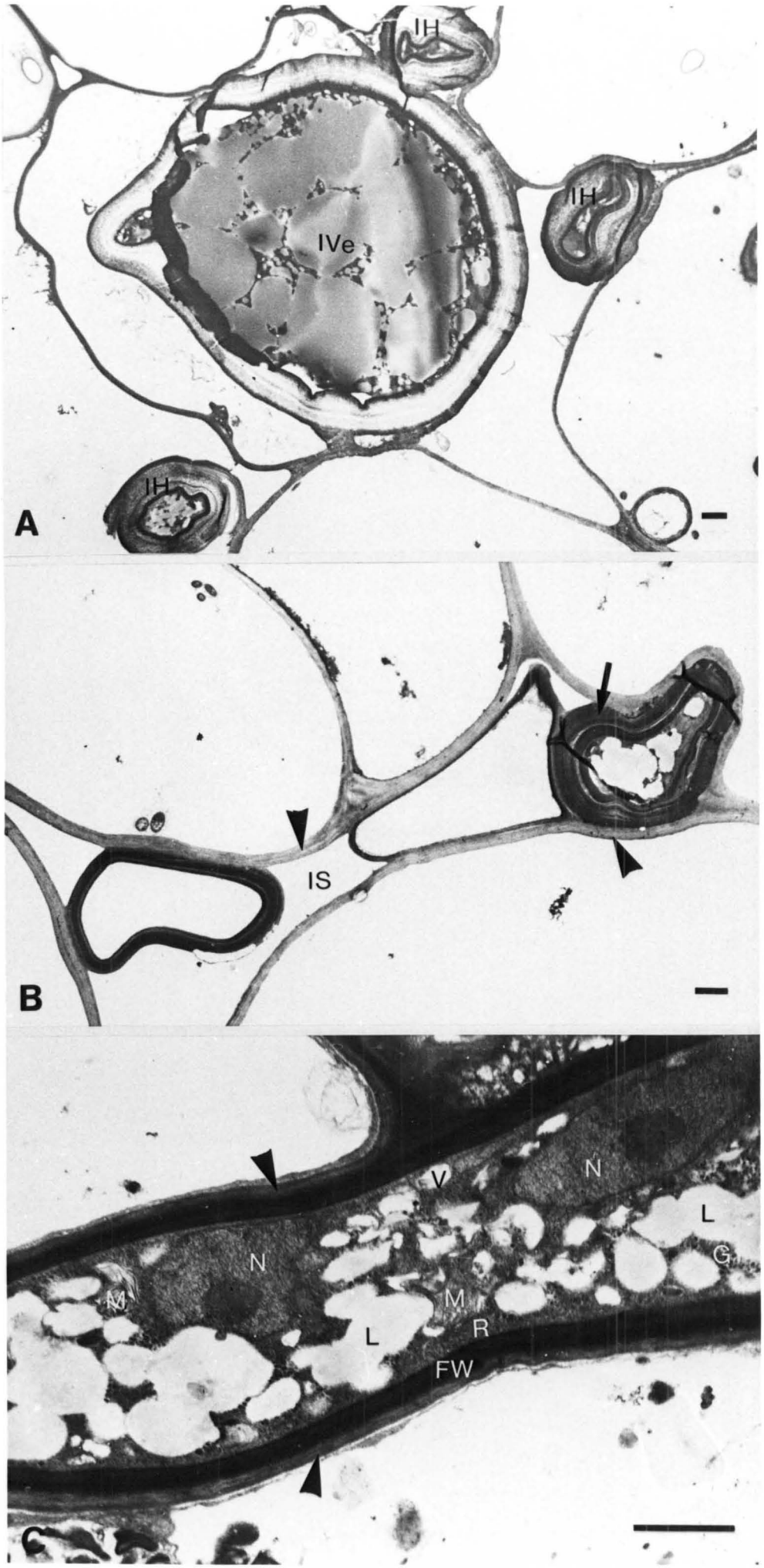
V - vacuole; N - nucleus; L - lipid globule;
G - glycogen bodies; FW - wall of hypha;
R - ribosomes; IS - intercellular space.

Host walls indicated by arrowheads.

A. Transverse section of the central cortex containing a matured intracellular vesicle (IVe) and thick walled intercellular hyphae (IH). The vesicle is considerably larger than the intercellular hyphae. x4,000.

B. Transverse section of the root cortex showing three intercellular hyphae. One of the hyphae (arrow) contains protoplasm and shows a considerably thicker wall than the other two hyphae which are devoid of content. x6,000.

C. Longitudinal section of cortex showing a young intercellular hypha containing a densely stained wall, dense cytoplasm with nuclei, ribosomes, glycogen bodies, small vacuoles, lipid globules and mitochondria. x17,000.


IH
IVe
IH
IH
A
IS
B
V
N
L
G
N
M
M
R
L
FW
C

develop and mature, vacuoles of various sizes appear in the cytoplasm (Plate 2.12C). The cytoplasm with its various organelles, surrounds the tonoplast of the vacuoles. At this stage, the hyphal wall appears as a single, darkly stained layer, lined on the inside by the plasmalemma (Plate 2.12 C, D). With further maturing of the hyphae, a lightly stained zone begins to appear on the inside of the dark stained zone facing the protoplast (Plate 2.12E). This inner zone has a convoluted margin bordering the protoplast with some prominent projections, suggestive of material having been recently incorporated into the developing wall (Plate 2.12E, F).

In some hyphae, the light staining inner zone continues to increase in thickness as it differentiates further (Plate 2.13A). At this stage, individual lamellae begin to be evident in the inner zone (Plate 2.13A) while the demarcation between the outer and inner zones becomes progressively less sharply defined (Plate 2.13B). Further differentiation of the hyphal wall results in the appearance of more lamellae in the inner zone and an increase in thickness of both the dark stained outer and the lighter stained inner zones (Plate 2.13C, D). The total thickness of the wall illustrated is about 1.0 μm, while the protoplast averages 2.0 μm in diameter. The walls of other such hyphae typically measure between 1.0 - 1.5 μm in thickness but some may reach up to about 2 μm (Plate 2.13E).

The thickness of the wall relative to the diameter of the enclosed protoplast also varies among the thick-walled hyphae. In some hyphae, the protoplast progress-

Plate 2.12 Ultrastructure of intercellular hyphae at various stages of development. Sections post-stained in uranyl acetate/lead citrate. Bar scale = 1.0 μm unless otherwise stated.

Abbreviations:

N - nucleus; M - mitochondrion; G - glycogen bodies; V - vacuole; FW - wall of hypha; OZ - outer zone of hyphal wall; IZ - inner zone of hyphal wall.

Host walls indicated by arrowheads.

A. Transverse section of a developing hypha containing a prominent nucleus. The cytoplasm is enclosed by a plasmalemma (curved arrow) and a thin wall (arrow). x26,000.

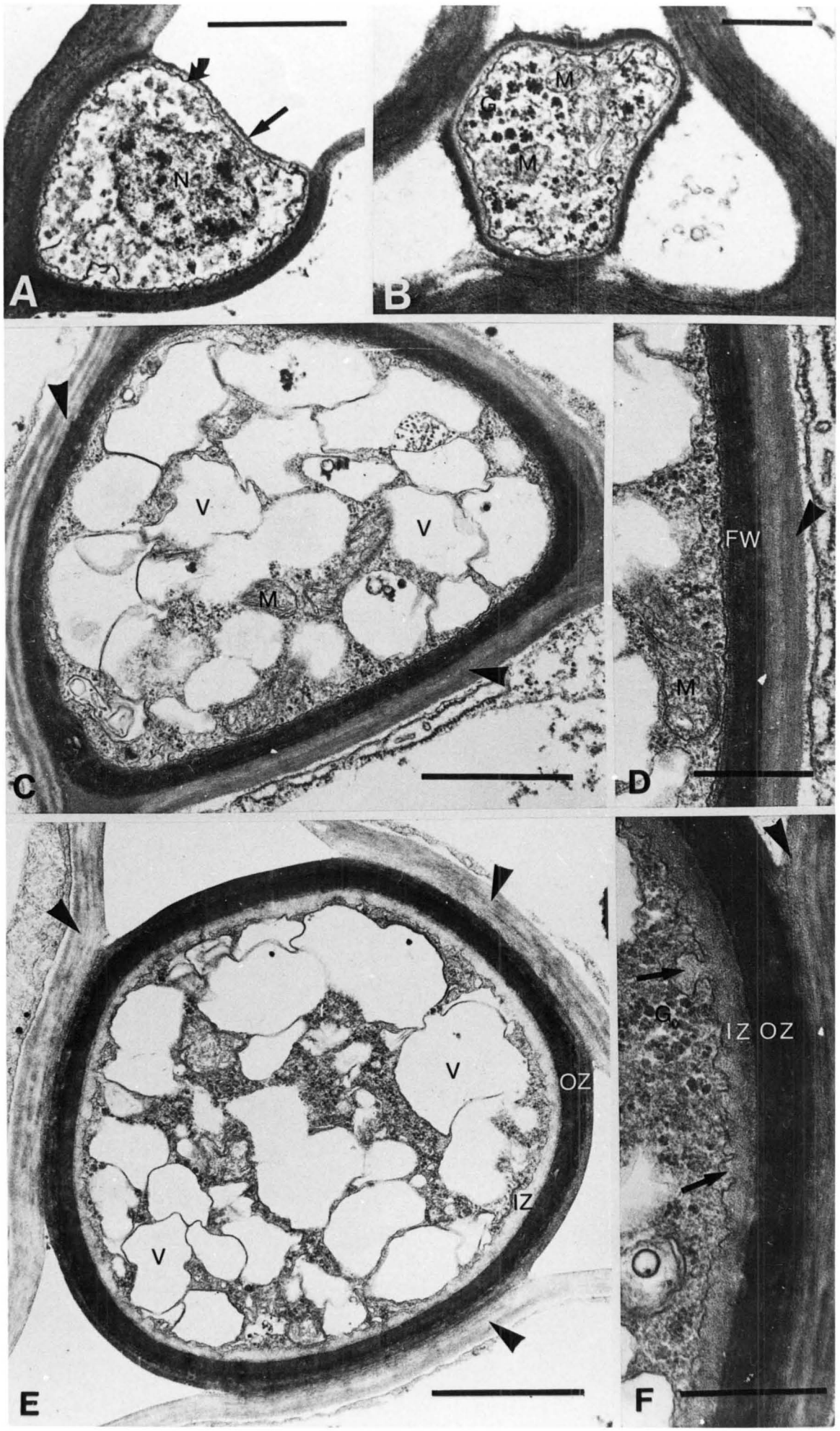
B. A young hypha with mitochondria, ribosomes and glycogen bodies in the cytoplasm. x34,000. Bar scale = 0.5 μm.

C. Transverse section of a hypha with a number of vacuoles in the cytoplasm. x28,000.

D. Portion of wall of the hypha shown in C, with the abutting wall of a host cell (arrowhead). The hyphal wall appears as a darkly stained zone at this stage of development. x44,000.

E. Transverse section of a hypha containing wall with a darkly stained outer zone and a lightly stained inner zone. x28,000.

F. Portion of wall of hypha shown in E. The lightly stained inner zone has a convoluted margin bordering the protoplast with some prominent projections (black arrows). x54,000.


A
N
B
M
G
M
C
V
V
M
D
FW
M
E
V
OZ
IZ
V
F
G
IZ OZ

ively loses its structural organization and reduces in volume through the continued centripetal growth of the wall (Plate 2.13D, E). In extreme cases, the protoplast may become almost completely occluded (Plate 2.13F). The walls of these hyphae appear to show a more gradual transition in staining from the outer to the inner layers (Plate 2.13E, F). Other thick-walled hyphae may show alternating dark and light staining bands (Plate 2.14A, C), sometimes of uneven widths (Plate 2.14A), throughout the thickness of the wall.

The fungal wall showed a strong positive reaction for polysaccharide material after PA-TCH-AgPr treatment (Plate 2.14A, B; 2.15E). The walls of surrounding host cells also reacted positively but the intensity of staining was less. Polysaccharide material was identified throughout the thickness of the fungal wall, with the highest concentrations corresponding to those regions staining heavily with uranyl acetate and lead citrate (cf. Plate 2.14C and D). No evidence was obtained for the presence of impregnating compounds (e.g. melanins) in these thick walls. The walls of hyphae and host cells did not give a positive reaction in the various controls for PA-TCH-AgPr treatments.

The gross layering of the fungal wall was shown, from high magnification studies, to result from aggregations of individual lamellae (Plate 2.14C). The lamellae appear to be the basic architectural component of the wall. Each lamella consists of a close aggregation of microfibrillar components, mostly aligned in the tangential plane.

Between the lamellae, microfibrils are fewer and less orderly arranged; such regions constitute the lighter stained parts of the wall (Plate 2.14C). The dark staining bands of the wall are where the lamellae are closely packed and later the lamellae may no longer be discernible individually. In transverse section, the lamellae typically appear as concentric rings (Plate 2.13D, F; 2.14A) and in longitudinal section as parallel bands (Plate 2.14B); three dimensionally such an arrangement would approximate concentric cylinders of lamellae within the wall.

An unusual feature associated with these thick-walled intercellular hyphae is the presence of broken layers in the wall. These occur in the outer region (Plate 2.13D, E) but frequently also in layers more deeply situated in the wall (Plate 2.15B, D). Some hyphae show breaks with a small separation of the broken layers (Plate 2.13D; 2.15A). Others have deep breaks with widely disjunct layers (Plate 2.15B, C, D, E). Where a wide separation occurs, the underlying layers remain continuous, but show a pronounced local thickening beneath the break (Plate 2.15B, C, D, E). Adjacent to where the break occurs, the wall sometimes shows an eroded appearance (Plate 2.15 B, D). This erosion is also found in other regions of the intact walls in some hyphae (Plate 2.15A, D, E), and is not an artifact of the fixation process. The eroded regions are either less intensely stained than intact walls or give a negative reaction for polysaccharides (Plate 3.15F).

Plate 2.13 Transverse sections of intercellular hyphae at more advanced stages of development. Sections post-stained in uranyl acetate/lead citrate. Bar scale = 1.0 µm.

Abbreviations:

OZ - outer zone of hyphal wall; IZ - inner zone of hyphal wall; P - protoplast.

Host walls indicated by arrowheads.

A. A hypha with a thicker inner zone than the hypha shown in Plate 2.12E. The individual lamellae of the inner zone are becoming discernible. x31,000.

B. A hypha showing a less sharply defined demarcation between the outer and inner zones of the wall. x25,000.

C. Hypha with a multilamellated wall. The lamellae of the darkly stained zone are more closely packed than those in the lighter stained zone. x15,000.

D. A later stage in development of hyphal wall. Both the outer and the inner zones of the wall have thickened compared to earlier stages shown in A to C. The outermost wall layers have breaks in them (arrows). Note the convoluted margin of the wall bordering the darkly stained condensed protoplast. x15,000.

E. Hypha at a further stage of wall differentiation and protoplast reduction than that shown in D. Note the ruptured outer wall layers. x15,000.

F. Hypha where the lumen has become almost completely occluded through continued centripetal

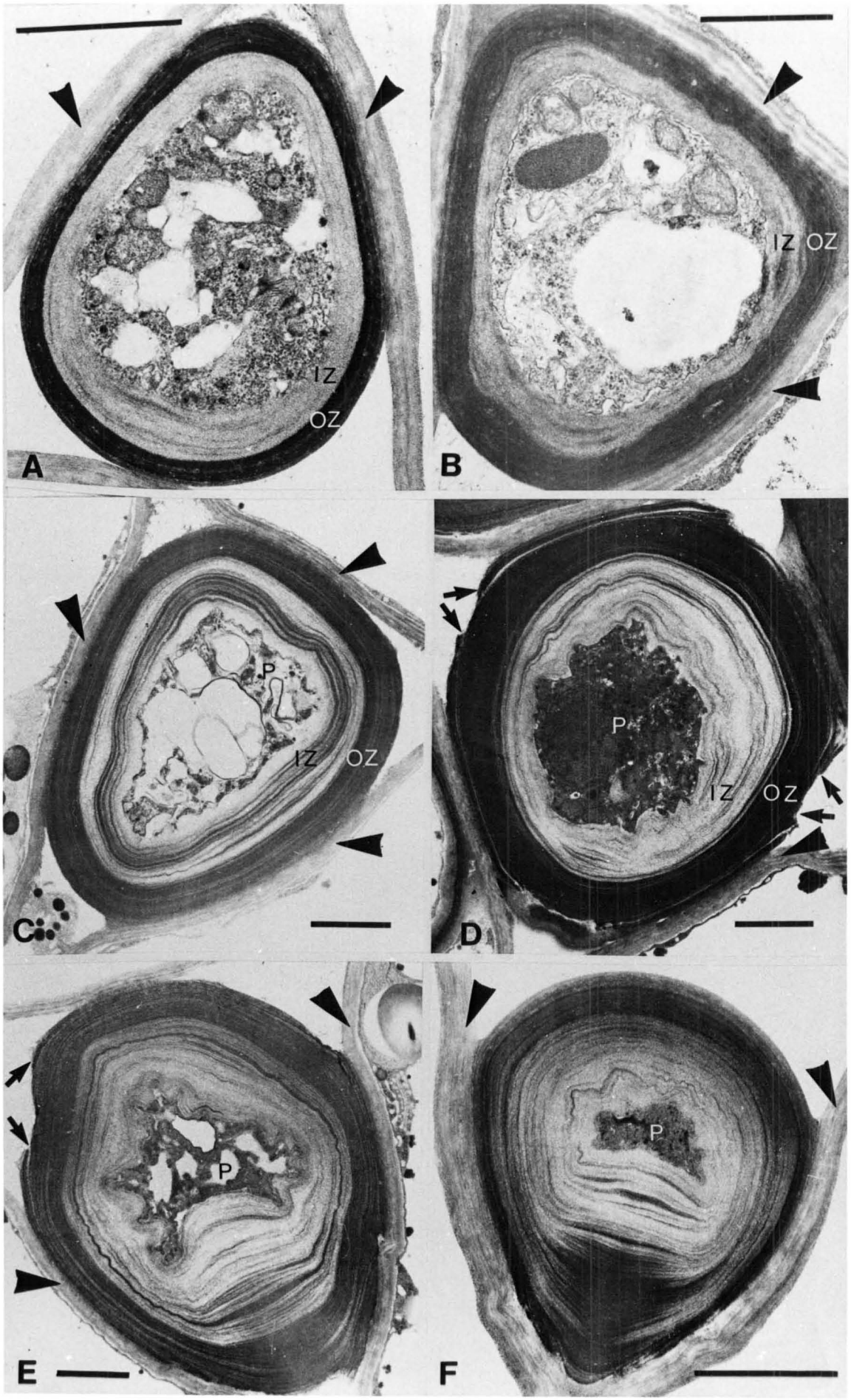

A
IZ
OZ
B
IZ OZ
C
P
IZ OZ
D
P
IZ OZ
E
P
F
P

Plate 2.14 Detail ultrastructure of thick walls of intercellular hyphae of *G. fasciculatus*. Sections A, B, D treated with PA-TCH-AgPr. Section C post-stained in uranyl acetate/ lead citrate. Bar scale = 1.0 μm unless otherwise stated.

Abbreviations:

G - glycogen bodies; P - protoplast; FW - wall of hypha.

Host walls indicated by arrowheads.

A. Transverse section of a hypha showing positive reactions to PA-TCH-AgPr treatment of the hyphal wall, glycogen and polysaccharide containing bodies in the cytoplasm (arrows). x23,000.

B. Longitudinal section of hypha showing alternating dark and light staining bands and the lamella organization of the wall. x31,000.

C. Transverse section of thick wall of hypha showing closely packed lamellae of microfibrils in the dark staining bands and more widely spaced lamellae in the lighter staining bands. An electron translucent zone (white arrowhead) occurs between the primary (PW) and the secondary wall. Note the loose microfibrils in the wall close to the protoplast (asterisk). x50,000.

D. Portion of wall of a hypha comparable to that shown in C, but treated with PA-TCH-AgPr to demonstrate the location of the polysaccharide material. x47,000. Bar scale = 0.5 μm.

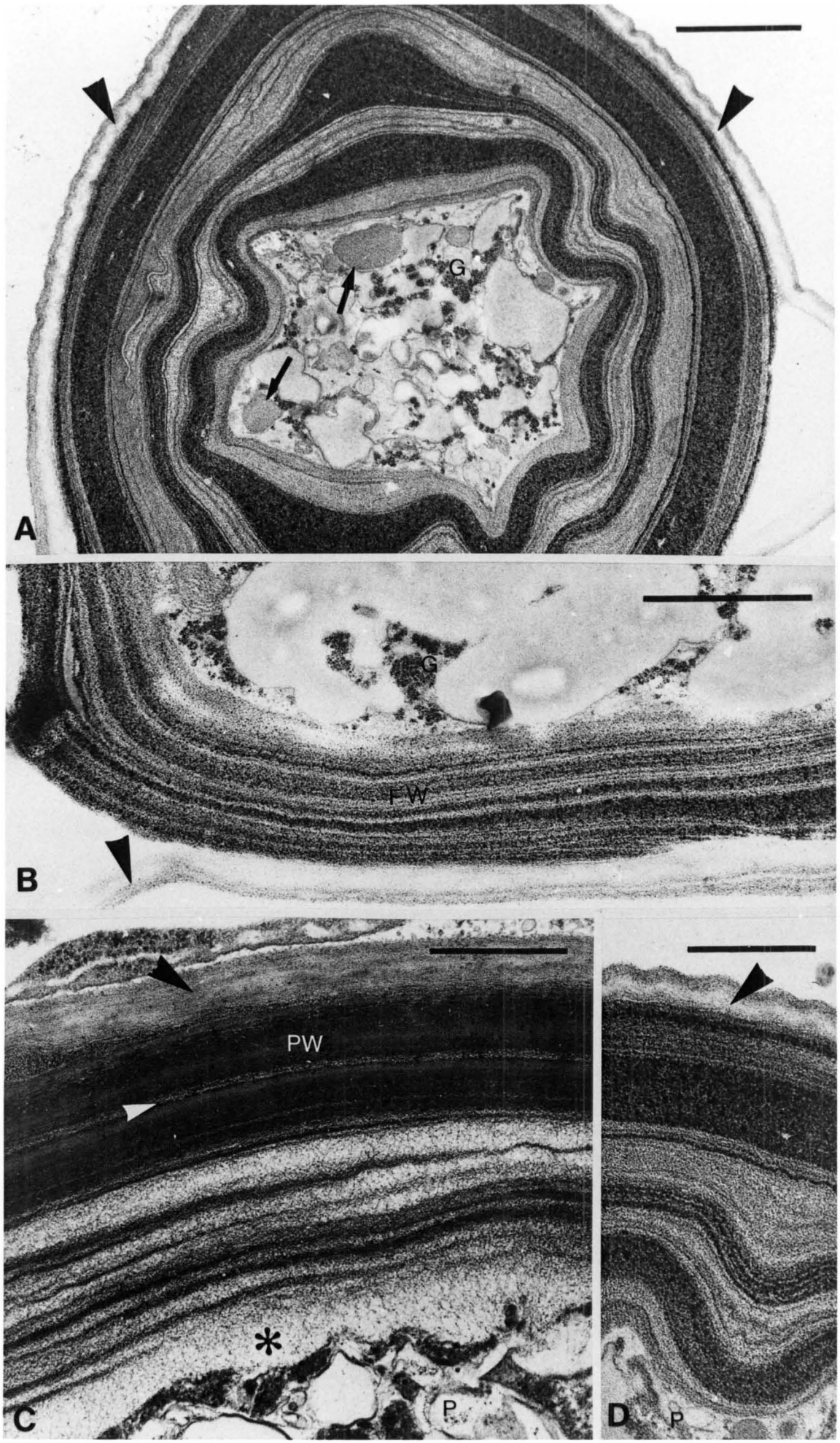

A
G
B
G
FW
C
PW
*
P
D
P

Plate 2.15 Transverse sections of thick-walled intercellular hyphae showing the phenomenon of broken layers in the wall. Sections A to D post-stained in uranyl acetate/lead citrate. Sections E and F treated with PA-TCH-AgPr. Bar scale = 1.0 μm.

Abbreviations:

P - protoplast.

Host walls indicated by arrowheads.

A. Hypha showing rupture of outer wall layers (arrow) and a small separation of the ruptured layers. Regions with eroded appearance indicated by white arrows. x18,000.

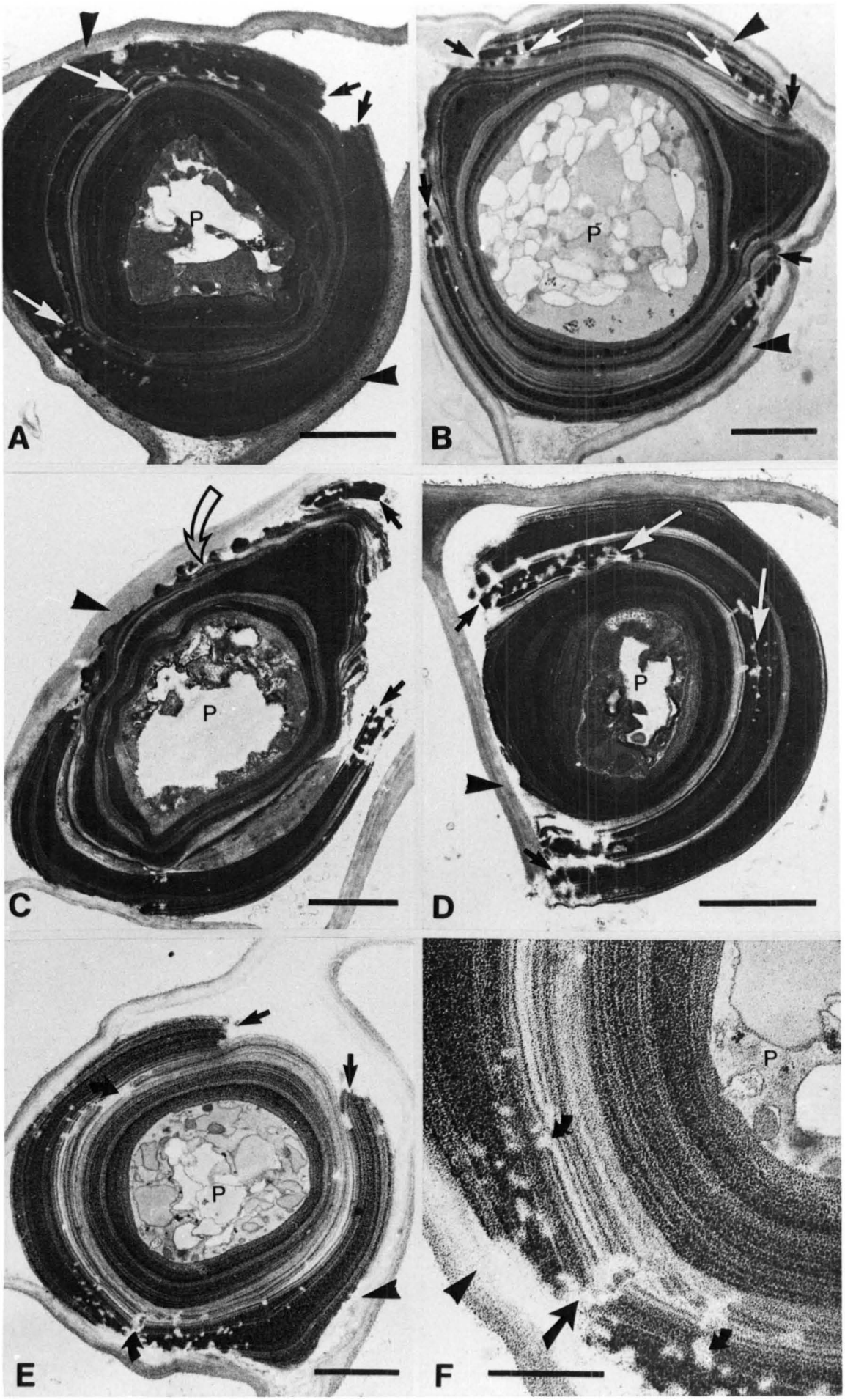
B. Hypha showing a local thickening of wall beneath the two regions of break of the outer wall (arrows). Wall layers adjacent to the breaks appear eroded (white arrows). x16,000.

C. Oblique transverse section of hypha showing severely eroded appearance of the inner wall (open curved arrow) adjacent to the ruptured wall layers (arrows). x17,000.

D. Hypha showing a wide separation of the ruptured outer wall layers (arrows) and eroded appearance at other regions of the wall (white arrows). x22,000.

E. Hypha with a wide separation of the outer ruptured wall layers (arrows) and also areas showing initial stages of break (curved arrows). x16,000.

F. Portion of wall of hypha shown in E. The eroded areas (curved arrows) and the slight break (arrow) in the lamellae show no staining with PA-TCH-AgPr treatment or appear less intensely stained than the adjacent areas. x43,000.


A
P
B
P
C
P
D
P
E
P
F
P

Another unusual feature observed in the intercellular hyphae of *G. fasciculatus* was the development of intrahyphal hyphae. These occur within hyphae which have a thin, single layered wall (Plate 2.16A, D; 2.17A) and in thick walled hyphae containing two or more wall layers (Plate 2.16B, C; 2.17D).

Remnants of the protoplasts of the 'parent' hyphae are seen between the inner hyphae and the original hyphae. They appear condensed or degenerated, with little recognizable structural organelles (Plate 2.16A, D; 2.17C, D). The size of the intrahyphal hyphae in relation to the intercellular hyphae varies. In some cases, intrahyphal hyphae appear distinctly smaller (Plate 2.16D; 2.17A, C, D). In other cases, they are almost equal in size, with the wall of the inner hypha closely appressed to the wall of the outer hypha in some regions (Plate 2.16B, C).

Intrahyphal hyphae vary also in the thickness of their walls, some having a thin, darkly staining wall (Plate 2.16A, B, C) and others considerably thicker walls (Plate 2.16D). The walls of the intrahyphal hyphae show a positive reaction when treated with PA-TCH-AgPr (Plate 2.17A, C). In some cases, the wall shows a darkly staining inner zone and a lighter staining outer zone (Plate 2.17D). In these cases, the walls of the outer hyphae also show a similar differential staining reaction of the outer and inner zones. The protoplasts of the intrahyphal hyphae also vary from those that appear metabolically active (Plate 2.16A, B) to those at various stages of degeneration (Plate 2.16C, D; 2.17D).

Plate 2.16 Intrahyphal hyphae development in intercellular hyphae of *G. fasciculatus*. Sections post-stained in uranyl acetate/lead citrate. Bar scale = 1.0 µm.

Abbreviations:

N - nucleus; V - vacuole; L - lipid globule; G - glycogen bodies; P - protoplast of outer hypha; IP - protoplast of inner hypha; FW - wall of hypha.

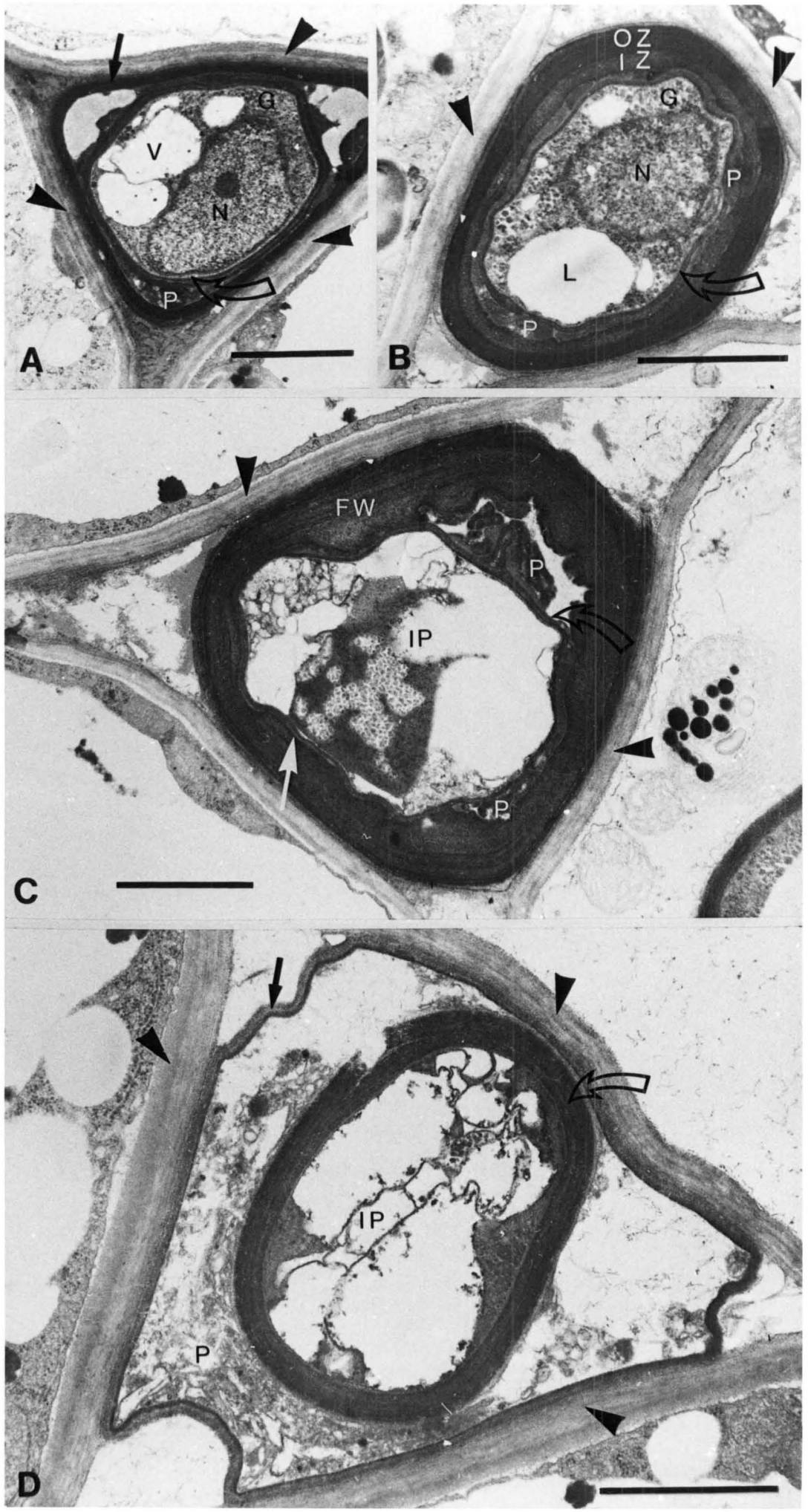
Host walls are indicated by arrowheads.

A. Transverse section of a thin-walled hypha (arrow) containing another hypha (open curved arrow) with metabolically active protoplast. Note the protoplasmic remnants of the outer hypha present in between the walls of the two hyphae. x21,000.

B. Thin-walled intrahyphal hypha (open curved arrow) within an intercellular hypha containing a thick wall with an outer (OZ) and an inner zone (IZ). x26,000.

C. Intrahyphal hypha with a thin wall closely appressed at same regions to the inner wall of the outer hypha (white arrow). Protoplasmic remnants of the outer hypha are still evident at some regions between the walls of the two hyphae. x24,000.

D. A thick-walled intrahyphal hypha (open curved arrow) within a large thin-walled intercellular hypha (arrow). Protoplasts of both the hyphae appear degenerated. x31,000.


A
V
G
N
P
B
OZ
IZ
G
N
P
L
P
C
FW
P
IP
P
D
IP
P

In most cases of intrahyphal hyphae development, a single hypha develops within another hypha. Multiple intrahyphal hyphae development, however, also occurs occasionally and appears to be of two types. One involves the development of two intrahyphal hyphae successively within the original intercellular hypha (Plate 2.17B). In other instances the presence of three hyphae within the same intercellular hypha (Plate 2.17E) is seen. Longitudinal sections of hyphae showing intrahyphal hyphae were not frequently obtained. In one case, however, a fine hypha containing a septum within a thick-walled intercellular hypha was observed in longitudinal view (Plate 2.17E).

2.4 DISCUSSION

The general features of infection of *G. fasciculatus* in white clover roots are very similar to those produced by the same fungus in *Z. mays* (Gerdemann, 1965) and *O. umbellatum* (Bonfante-Fasolo & Scannerini, 1977). They differ, however, from the situation in *L. tulipifera* (Gerdemann, 1965) where the infection is almost entirely intracellular. As in some other VA mycorrhizal associations (see Gerdemann, 1968), the apical meristems, endodermis and vascular tissues of white clover roots are not infected by *G. fasciculatus*. Infection in the epidermis and outer cortex is usually scanty.

Ultrastructural studies show that the development and sequential degeneration of the arbuscules of *G. fasciculatus* in white clover roots does not differ greatly from

Plate 2.17 Intercellular hyphae of *G. fasciculatus* showing single or multiple intrahyphal hyphae development. Transverse sections A to E treated with PA-TCH-AgPr. Longitudinal sections F post-stained in uranyl acetate/lead citrate. Bar scale = 1.0 µm.

Abbreviations:

P - protoplast of outer hypha; IP - protoplast of intrahyphal hypha; S - septum.

Host walls indicated by arrowheads.

A. Hypha (arrow) containing an intrahyphal hypha with wall (open curved arrow) showing uniform staining reactions to PA-TCH-AgPr. x34,000.

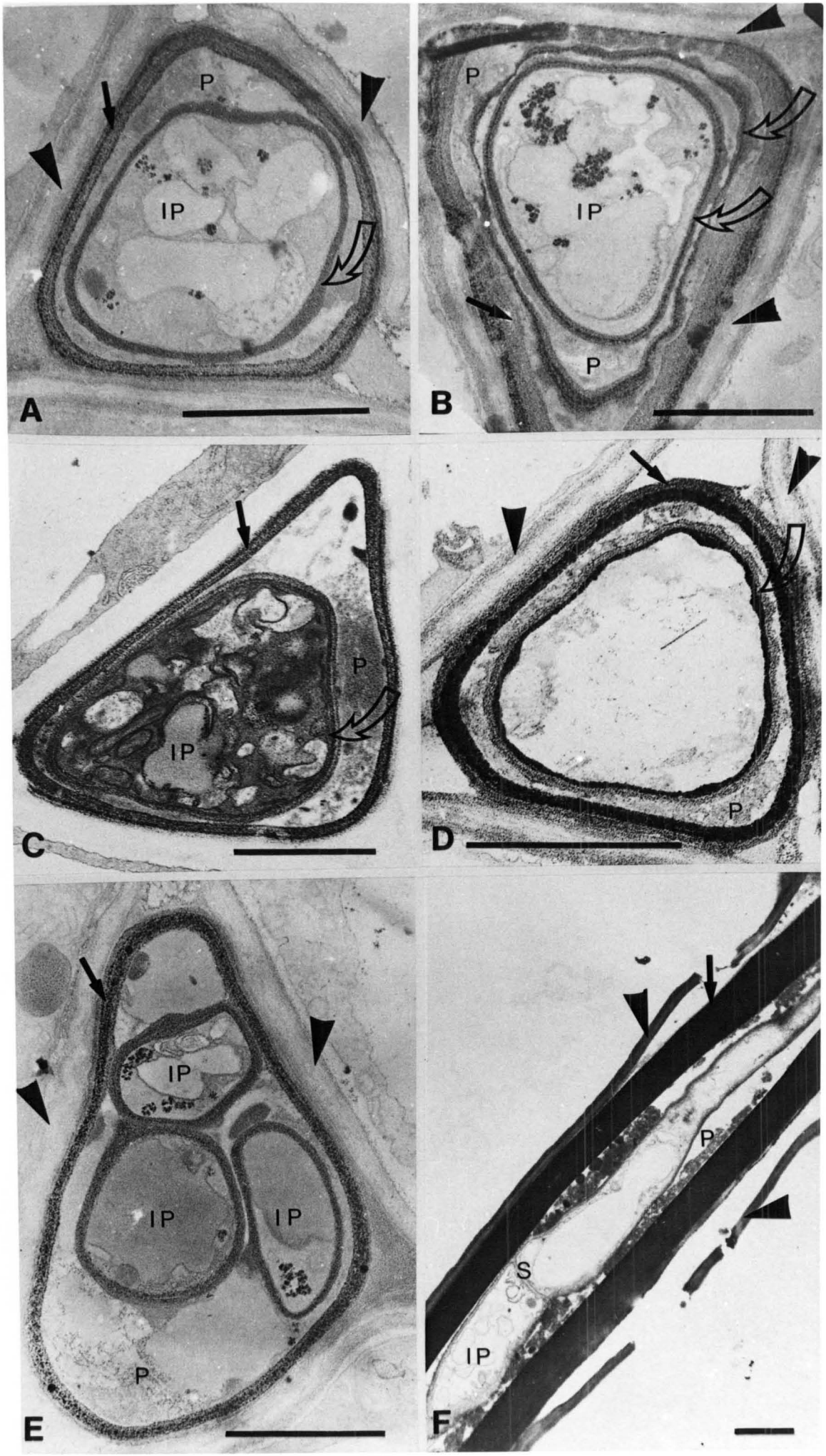
B. Successive development of two intrahyphal hyphae (open curved arrows) within a hypha (arrow). x29,000.

C. Hypha (arrow) containing an intrahyphal hypha (open curved arrow) with a dark stained inner and a lightly stained outer zone in its wall. x26,000.

D. An inner hypha almost devoid of content (open curved arrow) within an intercellular hypha (arrow). The walls of both the outer and the inner hyphae show a similar darkly stained inner and a lightly stained outer zone after PA-TCH-AgPr treatment. x39,000.

E. Three intrahyphal hyphae within an intercellular hypha (arrow) of *G. fasciculatus*. x29,000.

F. Longitudinal section of a thick-walled intercellular hypha (arrow) containing a septate inner hypha with thin walls. x11,000.


P
IP
A
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IP
P
B
P
IP
C
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D
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P
E
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S
IP
F

some other VA mycorrhizae (Cox *et al.*, 1975; Cox & Sanders, 1974; Holley & Peterson, 1979; Kinden & Brown, 1975c, 1976). The presence of an interfacial matrix between the arbuscule walls and the host plasmalemma observed in this study has also been noted in other VA mycorrhizal associations (Bonfante-Fasolo & Scannerini, 1977; Dexheimer *et al.*, 1979; Kinden & Brown, 1975c). It appears to be a consistent host response to intracellular development of VA mycorrhizal fungi in the host roots.

In this study, the continuity of the interfacial apposition layer from the host wall around the arbuscular trunks suggests its probable host origin. This is supported by similarities of the apposition layer and the host walls in texture and staining reactions when post-stained with uranyl acetate/lead citrate, and especially when treated with PA-TCH-AgPr. The interfacial matrix in the VA mycorrhiza of *O. umbellatum*, caused by the same fungus, has also been shown by Scannerini and Bonfante-Fasolo (1979) to be made up of wall material from the host cell, using various cytochemical tests. It is suggested to contain a glycoprotein complex and to diminish and change in nature with development of the finer arbuscular branches (Bonfante-Fasolo *et al.*, 1981). The interfacial matrix around the smaller arbuscular branches in this study also appears different from the apposition material around the trunk hyphae, being apparently amorphous or fibrillar in nature.

The enlargement of nuclei and the abundance of organelles, such as the mitochondria, in the cytoplasm, but absence of starch, suggests an increase in metabolic activity

in cells containing developing or mature arbuscules. Similar observations have been made in other VA mycorrhizal associations (e.g. Cox & Sanders, 1974; Kinden & Brown, 1975c).

In sections treated with PA-TCH-AgPr, the walls of collapsed and aggregated fragments of the smaller branches are less intensely stained than those of intact arbuscular trunk or collapsed walls of larger branches. This seems to suggest that progressive degeneration, especially of the polysaccharide materials of the collapsed walls, is responsible for the eventual disappearance of the wall fragments. This observation differs from that made by Bonfante-Fasolo *et al.* (1981). They claim that the cytochemical characteristics of the living hyphae are retained by the collapsed aggregated fungal walls, although they appear less well-defined. This difference could have been due to the different stages of degeneration of the collapsed arbuscule walls in the two examples. The lighter staining wall fragments observed in this study are likely to be at a more advanced stage of degeneration and appear greatly compressed and distorted in shape.

Reports on the developmental stages of vesicles in VA mycorrhizae are lacking due to the difficulties in obtaining satisfactory ultrathin sections (Kinden & Brown, 1975b). In most of the published ultrastructural work on VA mycorrhizae where reference is made to vesicles, only the ultrastructure of matured vesicles is shown. Here the ultrastructural features of younger, developing vesicles are illustrated. The cytoplasm of these vesicles usually

contains abundant organelles. Development and maturation of the vesicles appears to cause a thickening of the vesicle wall, an increase in the number of lipid globules and their coalescence into larger lipid bodies. The other organelles become less distinct as the cytoplasm condenses. Walls of mature vesicles appear similar to the thickened walls of some intercellular hyphae in their multilaminate nature of alternating dark and light staining bands. The walls of vesicles in other VA mycorrhizal associations so far reported all appear to be trilaminate (Bonfante-Fasolo & Scannerini, 1977; Holley & Peterson, 1979; Kinden & Brown, 1975b). Furthermore, the walls of mature inter- and intracellular vesicles of *G. fasciculatus* in white clover are thick, while the intracellular vesicles of *G. fasciculatus* in *O. umbellatum* apparently only have thin walls (Bonfante-Fasolo & Scannerini, 1977), even in the mature stage. It appears that differences do occur in the detailed ultrastructural features of the same VA mycorrhizal fungus growing in different host plants.

The ultrastructural features of young intercellular hyphae of *G. fasciculatus* in white clover roots are similar to other VA mycorrhizae (e.g. Cox *et al.*, 1975; Scannerini, *et al.*, 1975). Hyphae from old portions of the host roots, however, exhibit some features that have not been reported in ultrastructural studies of intercellular hyphae of other VA mycorrhizal associations. Many of these hyphae have considerably thickened walls, which in some instances, lead to the near occlusion of the protoplast. In some cases, the outer lamellae of these thick-walled hyphae are ruptured

to varying degrees, presumably during the phase of extensive deposition of inner wall material. Other hyphae which may have thick or thin walls, show the presence of intrahyphal hyphae.

Thick walls are normal features of fungal spores, but uncommon among vegetative hyphae (Waters *et al.*, 1972). Uniformly thickened walls have been reported in medullary cells of sclerotia (e.g. Waters *et al.*, 1975) and differentially thickened walls in vegetative hyphae of *Phytophthora* (Gooday & Hunsley, 1971). Fibre hyphae in rhizomorph strands of *Armillaria mellea* and *Trametes quercina* similarly have thickened walls (Schmid & Liese, 1968). With mycorrhizal associations, thick walls sometimes occur among hyphae external to the host root (Nicolson, 1959), but there has only been incidental mention of thick walls among hyphae within the root tissues. Bonfante-Fasolo and Scannerini (1977) showed a few thickened walls in the VA mycorrhiza in *O. umbellatum*, but these walls were not particularly thick relative to the diameter of the hyphae. The walls of some intercellular hyphae of *G. fasciculatus* found were between 1.5 and 2.0 µm in thickness, thicker than the wall of the extraradical hyphae of *G. fasciculatus* (about 0.30 - 0.50 µm thick) reported in *Vitis vinifera* by Bonfante-Fasolo and Grippiolo (1982).

The walls of growing hyphae of most fungi are typically not very thick (often 0.2 µm or less) but they may thicken after their formation, even near the growing tips (Burnett, 1976). However, secondary wall deposition is uncommon in hyphae and is seldom extensive if it occurs. In this study,

an inner secondary wall is differentiated in some hyphae, leading to extensive deposition of wall material in some instances.

The mechanism for the deposition of secondary wall material is not known. The presence of an irregular profile of the plasmalemma, at least during the initial stages of differentiation, suggests the incorporation of vesicle containing wall material or its precursors. Sections treated with PA-TCH-AgPr sometimes showed single membrane bounded vesicles near the plasmalemma containing polysaccharide material resembling that of the wall. The darkly stained outer zone of thick-walled hyphae probably represents the primary wall developed during penetration and establishment of the fungus in the cortex. In some cases, the demarcation between the outer and inner zones becomes indistinct probably as a result of compaction on the underlying secondary wall material.

Thick walls of *G. fasciculatus* with broken disjunct outer wall layers have not been reported in ultrastructural studies of VA mycorrhizal fungal hyphae. The local thickenings of the inner wall layers beneath the breaks may correspond to the angular projections described in optical microscopic sections in this and other studies (e.g. Gerdemann, 1955). The main factor responsible for the rupturing of the wall is likely to be the pressure exerted by the growing hypha as it continues to lay down secondary wall material centripetally or as local thickenings. The presence of eroded areas adjacent to the breaks suggests that some local enzymic activity may be involved. The walls are probably

weakened by enzymic activity prior to their actual separation by the mechanical force exerted by local thickenings.

The lamellate organization of the thick walls of *G. fasciculatus* intercellular hyphae is revealed by high magnification studies. Bonfante-Fasolo (1982), in a study employing cryoultramicrotomy, showed that the wall of intercellular hyphae of *G. fasciculatus* in *O. umbellatum* were constantly of an amorphous texture. In her work, the walls of hyphae examined were all relatively thin, measuring between 0.07 µm (Fig. 3) to 0.24 µm (Fig. 5) and therefore not comparable to the wall thickness of the thick-walled hyphae found in this study, which measure between 0.3 to 2.0 µm in cases where the inner zone is present. The walls of hyphae illustrated in her study probably correspond only to the primary wall layer of the hyphae observed here.

Recently, Bonfante-Fasolo and Grippiolo (1982) reported the occurrence of multilamellate structures in the walls of both the extraradical and coiled hyphae of *G. fasciculatus* in the outer cortex of grapevine roots. The walls of the intercellular hyphae are thin (about 0.12 µm) and are monostratified. Again, these intercellular hyphae probably correspond in their developmental stages to those in this study whereby only the primary wall layer is present. The mycorrhizal roots used by them were two months old, whereas those used in this study were harvested six months after inoculation. It appears that a multilamellate secondary wall is a feature of intercellular hyphal development.

Thick-walled hyphae occur commonly in older portions of the roots and appear to be connected to thick-walled inter- or intracellular vesicles and to arbuscular trunk hyphae in cells where the smaller branches have degenerated. Their common occurrence suggests that they could be important components in the life history of the endophyte and tempts the forwarding of hypotheses for their function.

It has become accepted for higher plants that the wall can provide an important route for the apoplastic transport of solution within the tissue (Lüttge & Higinbotham, 1979). This involves the primary walls of young cells and secondary walls where lignification has not taken place. An example of the apoplastic pathways for transport is seen in the glands of the carnivorous plants *Utricularia monanthos* (Fineran & Gilbertson, 1980). Here, the apoplastic pathway is confined to the non-impregnated regions of the secondary wall which contain polysaccharide. The multilaminate wall construction shown by *G. fasciculatus* is not dissimilar to the laminate organization now being recognized in the walls of higher plants (Roland & Vian, 1979), and in particular, to the extensive secondary wall developed in the external glands of *Utricularia* (Fineran & Lee, 1980). The densely stained and contracted protoplasts also appear similar in both examples, a condition having developed presumably as a consequence of centripetal deposition of extensive wall material. As the thickened walls of *G. fasciculatus* hyphae are also unimpregnated, they might provide a comparable pathway for transport of solution within the hyphal system, possibly from the surrounding tissue of the host cortex to the more actively metabolizing hyphae of the

endophyte. Transport along the length of the hyphae, could presumably be achieved by suitable concentration gradients established by these hyphae, rather than by the often senescent protoplasts of the very thick-walled hyphae themselves.

The role of thick-walled fungal hyphae in assisting water or solute transport has been suggested by other workers but they are given a support function for the translocating vessels rather than acting as the main translocating route. Duddridge *et al.* (1980), were of the opinion that in the rhizomorphs of ectomycorrhizae of *Pinus sylvestris*, water moves apoplastically through the large central 'vessel' hyphae lacking cytoplasmic contents, rather than symplastically through the narrow outer hyphae or through the cell wall. One function of these outer thick-walled hyphae appears to be for providing external support to the rhizomorphs. Jennings and Watkinson (1982) observed numerous thick-walled hyphae with occluded lumina around the edge of the mycelial strands of *Serpula lacrimans*, their presence was suggested to provide support to the matrix of the strands, consequently enabling translocation to take place in the central channel hyphae by means of pressure driven mass flow.

Another possible role for the thick-walled intercellular hyphae of *G. fasciculatus* is that they may serve as resistant vegetative propagules. Tommerup and Abbott (1981) reported that various mycorrhizal species, including *G. fasciculatus*, can renew their growth from hyphae in the cortex of roots which have been stored under adverse conditions at a matric water potential of approximately

-50 MPa for six months or longer. This suggests that internal hyphae of *G. fasciculatus* can remain viable after drying and act as propagules for reinfection as the conditions become favourable for growth. The development of thick walls could possibly be the mechanism by which this survival is maintained. However, the apparent lack of impregnating compounds common to dormant spores (e.g. melanins) casts doubt on the resistant nature of these hyphae. Presence of lipid material in the chlamydospore walls of *Fusarium oxysporum* has been suggested by Griffiths (1973) to enable them to withstand extremes of desiccation in the soil. Sporopollenin, the resistant material characterizing the exine of pollen grains of Spermatophytes and spores of Pteridophytes, has been demonstrated by Gooday *et al.* (1973) in the zygospore wall of *Mucor mucedo*. There is, at present, no specific cytochemical stain which will identify sporopollenin by microscopy (Gooday, 1981). It is possible that resistant substances may be present in the thick-walled hyphae, but not revealed by the cytochemical staining techniques used in this study.

The presence of abundant lipids in the cytoplasm or contracted cytoplasm of some thick-walled hyphae may enable them to maintain a positive water potential with respect to the surrounding tissues, thus maintaining the structure in a relatively dehydrated state (Hawker & Madelin, 1976). This feature might help maintain the hyphae in an osmotically stable but viable condition in an adverse environment and to renew growth as conditions become favourable.

Intrahyphal hyphae development within intercellular hyphae has been observed in optical microscopic studies of VA mycorrhizae. Cox and Sanders (1974), noted the growth of young hyphae inside collapsed intercellular hyphae of *Glomus mosseae* in *Allium cepa* roots and Tommerup and Abbott (1981) found new hyphae growing within old hyphae of various VA mycorrhizal fungi in the cortex of roots which had been dried and stored for six months before returning to normal growth conditions. Intrahyphal hyphae development, however, has not been shown at the ultrastructural level for coarse VA endophytes although it has been noted for the fine endophyte, *Glomus tenuis* (Gianinazzi-Pearson *et al.*, 1981). In this study, hyphae growing within old hyphae of *G. fasciculatus* were shown to occur, especially in the older portions of the roots.

The growth of intrahyphal hyphae has been reported for other fungi that do not form VA mycorrhizal associations. They frequently occur under certain physiological conditions. Calonge (1968) observed them in *Sclerotinia fructigena* when grown in liquid culture. Chan and Stephen (1967) found intrahyphal hyphae only in injured or old hyphae of *Linderina* spp. Hammill (1972), however, found that they are a normal constituent of hyphae of *Monotosporella sphaerocephala*. It is also suggested that they are concerned with the normal production of successive conidia by conidiophores.

The origin and cause of intrahyphal hyphae development is unknown. Empty collapsed hyphae separated by septa from adjoining functional hyphae with protoplasts were observed under the optical microscope in this study, suggesting

that intrahyphal hyphae probably grow from functional hyphae into the old hyphal lumen through the septa. It is possible that the degenerating protoplasts of the outer hyphae could serve as a food source for the growing inner hyphae. As the inner hypha matures, it could in turn be invaded by another hypha giving rise to multiple intrahyphal hyphae development (Plate 3.17B). In this instance, the walls of the two outer hyphae appear eroded at various regions, suggestive of the involvement of enzymes in degrading the old hyphal walls which may be able to serve as a nutrient source for the inner growing hypha.

The walls of the outer and inner hyphae give similar positive reactions when treated with PA-TCH-AgPr, indicating the presence of polysaccharide materials throughout the walls (Plate 3.17C, D). In some cases, the walls of both hyphae show a darkly stained inner zone, and a lightly stained outer zone (Plate 3.17D), indicating that the hyphae originate from the same fungus and are not hyperparasitic in nature.

Multiple intrahyphal hyphae development consisting of three smaller hyphae within one intercellular hypha was also observed (Plate 3.17E). Whether the three hyphae originate from branches of a single intrahyphal hypha or by invasion of three separate hyphae of smaller diameter, could only be determined by further investigation. Branching of a single intrahyphal hypha into several branches has, however, been observed for other fungi and appears to be of common occurrence (Chan & Stephen, 1967; Lowry & Sussman, 1966; Calonge, 1968). The invasion of dead intercellular hyphae of *G. fasciculatus* by hyphae of a different fungus was also noted

(Plate 3.17F). In this case, the fungus was septate and it had a very thin wall, which stained differently from the hyphae of *G. fasciculatus*. Hyphae of this type were also observed invading empty dead host cells after the arbuscules had degenerated, presumably utilizing both dead hyphae and host cells as a food source.

CHAPTER 3

GROWTH RESPONSES OF WHITE CLOVER TO APPLIED PHOSPHORUS AND *GLOMUS FASCICULATUS* INFECTION

3.1 INTRODUCTION

Vesicular-arbuscular mycorrhizal fungi have been shown to increase the growth and phosphorus uptake of white clover in soils with low available phosphorus (Crush, 1976; Hall *et al.*, 1977; Powell, 1976). Mosse (1972) and Powell (1977b) in comparing the effects of several mycorrhizal fungi in different soils on the growth of onion and clover respectively, came to the conclusion that the efficiency of a mycorrhizal fungus at stimulating shoot growth varies in different soils.

Various workers had studied the growth responses of plants to VA mycorrhizal infection in soils applied with a range of phosphorus levels. The beneficial growth effects of VA mycorrhizae generally decrease with increase in soil available phosphorus and may ultimately turn into detrimental effects (Cooper, 1975; Crush, 1973; Hall *et al.*, 1977; Mosse, 1973b). The level of soil phosphorus at which this occurs appears to be dependent on a number of factors among which is the type of soil used. Mosse (1973b) compared the growth of mycorrhizal and non-mycorrhizal onion plants in several soils applied with a range of phosphorus levels. In some soils, it was found that mycorrhizal plants showed growth increases at all the phosphorus levels. In other soils, growth increases occurred only at low levels of phosphorus, with growth depression at higher levels.

In this study, the growth responses of 'Grasslands Huia' white clover to inoculation with *G. fasciculatus* were investigated in two soils applied with a range of different phosphorus levels.

3.2 MATERIALS AND METHODS

3.2.1 Experiment 1. Tasman silt loam

Tasman silt loam used in this experiment was prepared and fumigated as outlined in Chapter 2. Soil analysis data is presented in Table 3.1.

Fumigated soil mixture was weighed into 350 g aliquots and potted into 10 cm 'Squat pots' standing each in a 10 cm petri dish. Basal nutrients in 10 ml solutions containing 84 mg K_2SO_4, 306 mg $MgSO_4.7H_2O$ and 0.3 mg $Na_2MoO_4.2H_2O$ were added to each pot of soil and allowed to dry. The soil in each pot was then thoroughly mixed as described previously, after adding 750 mg $CaCO_3$.

The preparation of white clover seedlings and mycorrhizal inoculum, the transplanting and inoculation of seedlings with *G. fasciculatus* and rhizobia was as outlined in Chapter 2.

Five soil phosphorus levels containing 0, 10.7, 37.5, 131.3 and 459.6 mg P/pot (equivalent to 0, 3.5, 12.3, 42.9 and 150.1 Kg P/ha on a surface area basis) were applied as 20 ml $Ca(H_2PO_4)_2.H_2O$ solution, 35 days after transplanting. A 5 x 2 factorial design was used, laid out in 14 complete randomised blocks in a growth cabinet at 20-25^{o}C with 16 h daylight. The treatments were:

P levels: 0, 10.7, 37.5, 131.3, 459,6 mg P/pot

Mycorrhizal: Inoculated or uninoculated

Replicates: 14

Shoot yields of plants were determined after harvesting shoots 15 mm above soil surface at days 35 and 70 after phosphate application. The shoots were dried in a 105^{o}C oven for 24 h, cooled and weighed. At the second harvest, two soil cores (10 mm diameter) were collected from each mycorrhizal inoculated treatment, one 5 mm away from the plant and the second 5 mm from the edge of the pots. Root segments collected from the soil cores were carefully washed free of soil, cleared and stained with lactophenol trypan blue according to the procedure given in Appendix 1. Five randomly selected root segments from each pot were mounted onto slides. The percentage of roots infected by *G. fasciculatus* was estimated microscopically by a modified technique of Hayman (1974).

As there was heterogeneity in the treatment variances, the data for shoot yields were analysed using linear comparison of means (Dr, I. R. Hall, pers. comm.). Data for mycorrhizal root infection were analysed using ANOVA. The magnitude of mycorrhizal response for each phosphorus treatment was expressed as the ratio of shoot yields of mycorrhizal inoculated to uninoculated plants, and its standard error (S.E.M.) calculated using the approximation:

$$\sqrt{\left(\frac{\text{Variance of Y}}{X^2}\right) + \left[\left(\frac{Y}{X^2}\right)^2 \times \text{Variance of X}\right]}$$

where e.g. the variance of $Y = \frac{\text{E.M.S. of Y}}{n}$

n = no. of replicates.

(Dr. Stuart Crosbie, pers. comm.).

These ratios were then compared using linear comparison of means.

3.2.2 Experiment 2. Pawson Hill silt loam

A yellow-brown earth belonging to the Pawson Hill silt loam set (N.Z. Soil Bureau, 1968) collected from Banks Peninsula was used. Air-dried soil was mixed with equal volume of a coarse and fine sand mixture. The soil-sand mixture was then fumigated with methyl bromide, weighed into 500 g aliquots in polyethylene bags and treated with basal nutrients and lime as for Tasman silt loam. Soil analysis data is presented in Table 3.5.

Germination of 'Grasslands Huia' white clover seeds, the transplanting of seedlings, inoculation with *G. fasciculatus* and rhizobia were as described in Chapter 2.

The five soil phosphorus levels used were 0, 9.2, 27.6, 82.8, 248.4 mg P/pot (equivalent to 0, 3, 9, 27, 81 kg P/ha on a surface area basis) applied as 20 ml $Ca(H_2PO_4)_2.H_2O$ solution to the soil, 49 days after seedlings were transplanted. The experimental design and growth cabinet conditions were similar to experiment 1. The treatments were:

P levels 0, 9.2, 27.6, 82.8, 248.4 mg P/pot

Mycorrhizal: Inoculated or uninoculated

Replicates: 14

Three harvests of shoots were made, at days 35, 70 and 95 after application of phosphorus. The shoots were dried and weighed as described previously. At the third harvest, randomly selected root segments obtained by the soil core method were assessed for mycorrhizal root infection according to the procedure used for experiment 1. The results of shoot yield, mycorrhizal root infection and magnitude of mycorrhizal response were analysed as in experiment 1.

3.3 RESULTS

3.3.1 Experiment 1. Tasman silt loam

Table 3.1 Chemical analysis of Tasman silt loam sand mixture before (U) and after (S) fumigation with methyl bromide.

	PH	Ca (µg/mℓ)	K (µg/mℓ)	Olsen P (µg/mℓ)	Mg (µg/mℓ)	N (%)	P retention (%)
U	5.5	75	12	5	7	0.19	29
S	5.5	75	20	7	10	0.25	28

Growth of white clover was greatly stimulated by inoculation with *G. fasciculatus* in Tasman silt loam. Total shoot yields of inoculated plants were significantly greater ($P < 0.001$) than uninoculated controls at all levels of applied phosphorus (Table 3.2).

Table 3.2 Effect of applied phosphorus on total shoot dry wt. (g) of white clover (harvests 1 and 2) inoculated (M) or left uninoculated (N) with *G. fasciculatus* and grown in Tasman silt loam.

Applied P (mg/pot)	Total shoot dry wt. (g)			
	M		N	
0.0	0.411	(0.046)	0.005	(0.026)
10.7	0.614	(0.051)	0.008	(0.025)
37.5	0.875	(0.057)	0.046	(0.030)
131.3	1.282	(0.098)	0.470	(0.084)
459.6	1.484	(0.123)	0.638	(0.080)

S.E.M. in parentheses

Shoot dry wts of inoculated plants were greater than uninoculated plants from P = 0 mg/pot to P = 459.6 mg/pot, at both harvests 1 and 2, when analysed separately (Fig. 3.1A, B). There was a significant linear component in the response of white clover to applied phosphorus in the mycorrhizal inoculated and uninoculated treatments at both harvests 1 and 2 ($P < 0.001$).

Data for the magnitude of the mycorrhizal response are presented in Table 3.3. There was a significant linear trend in the decrease of these values with increase in soil phosphorus levels for both harvests 1 and 2 ($P < 0.001$). The magnitude of mycorrhizal response showed a significant increase from harvest 1 to harvest 2 at applied phosphorus = 0 mg/pot ($P < 0.001$), and significant decreases at 131.3 and 459.6 mg P/pot ($P < 0.02$ and 0.001 respectively). Differences between harvests 1 and 2 at P = 10.7 mg/pot and 37.5 mg/pot are not significant.

Table 3.3 Magnitude of mycorrhizal response (M/N) at harvests 1 and 2 for white clover grown in Tasman silt loam.

	M/N			
Applied P (mg/pot)	Harvest 1		Harvest 2	
0.0	33.23	(6.56)	268.13	(59.75)
10.7	53.17	(11.02)	115.92	(46.36)
37.5	28.86	(8.76)	14.98	(5.25)
131.0	6.33	(1.77)	1.84	(0.37)
459.6	4.60	(0.85)	1.6	(0.25)

S.E.M. in parentheses

Magnitude of mycorrhizal response = M/N

M = shoot dry wt. of inoculated plants

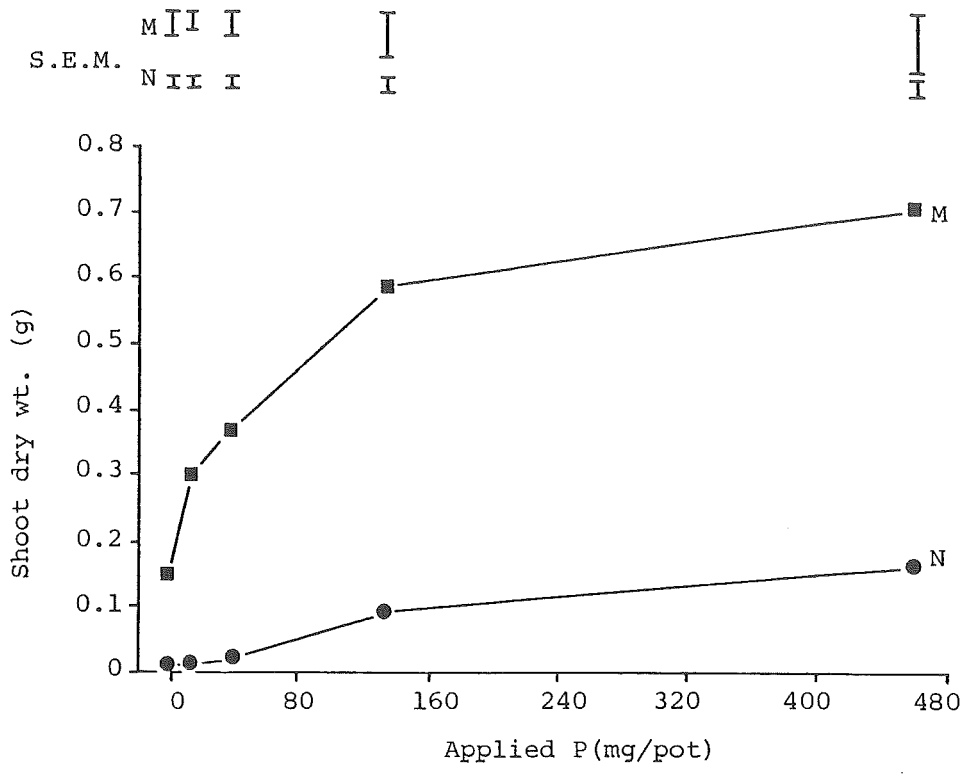
N = shoot dry wt. of uninoculated plants

Figure 3.1 Effect of applied phosphorus and presence (M) or absence (N) of *G. fasciculatus* on shoot dry weight of white clover in Tasman silt loam.

A. Harvest 1

B. Harvest 2

A. Harvest 1



B. Harvest 2

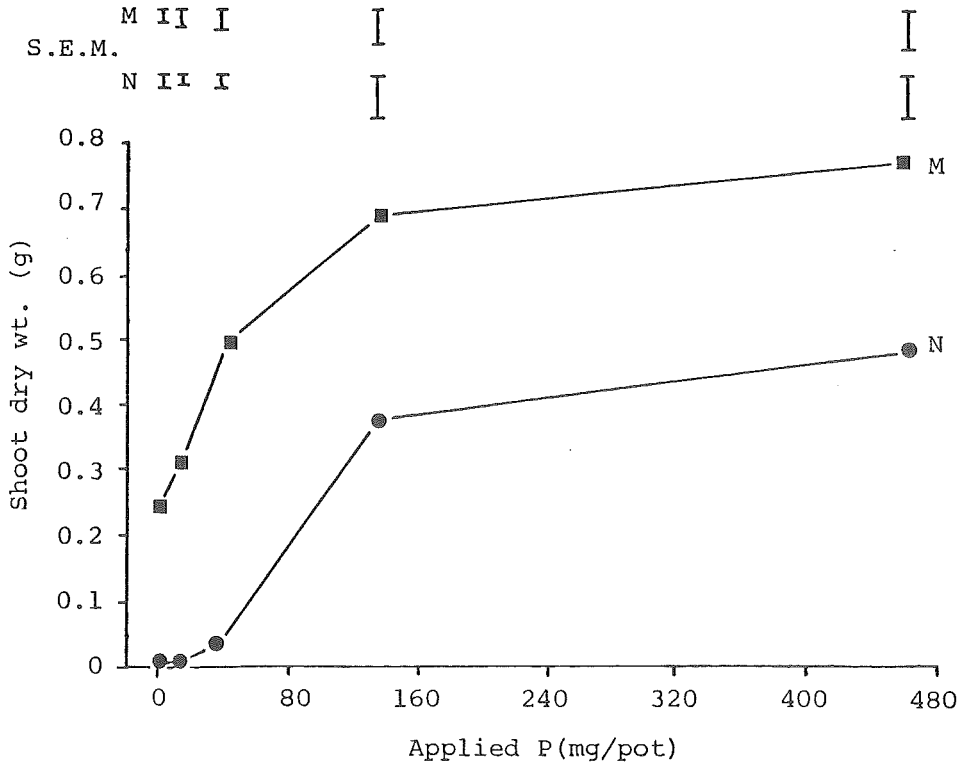


Table 3.4 shows the percentage of roots infected by *G. fasciculatus*. Percentage root infection decreased with increase in phosphorus levels. Percentage root infection at 0, 10.7, 37.5 mg P/pot were not significantly different, but those at 131.3 and 459.6 mg P/pot were significantly different from each other and significantly less than root infection at phosphorus levels less than 131.3 mg/pot.

Table 3.4 Percentage of roots infected by *G. fasciculatus* in Tasman silt loam at harvest 2.

Applied P (mg/pot)	Mycorrhizal Root Infection (%)
0.0	64.1
10.7	57.0
37.5	58.6
131.3	46.6
459.6	26.8

S.E.M. = 3.66

L.S.D. (5%) = 10.4

3.3.2 Experiment 2. Pawson Hill silt loam

Table 3.5 Chemical analysis of Pawson Hill silt loam before (U) and after (S) mixing with sand and fumigation with methyl bromide.

	PH	Ca (µg/mℓ)	K (µg/mℓ)	Olsen P (µg/mℓ)	Mg (µg/mℓ)	N (%)	P retention (%)
U	6.1	125	20	13	68	-	-
S	6.7	275	8	18	20	0.07	21.0

Growth of transplanted seedlings in Pawson Hill silt loam was initially very slow. The seedlings took a longer time to become established compared to the Tasman silt loam. Once established, the growth of seedlings became more comparable in the two soils.

Inoculation of white clover with *G. fasciculatus* increased their total shoot yield (P varying from <0.01 to <0.001) in Pawson Hill silt loam at each level of applied phosphorus, from 0 to 248.4 mg/pot (Table 3.6).

Table 3.6 Effect of applied phosphorus on total shoot dry wt. (g) of white clover (harvests 1, 2 and 3) with (M) and without (N) inoculation with *G. fasciculatus* in Pawson Hill silt loam.

	Total shoot dry wt. (g)			
Applied P (mg/pot)	M		N	
0.0	0.849	(0.069)	0.193	(0.053)
9.2	1.048	(0.061)	0.399	(0.749)
27.6	1.601	(0.114)	0.701	(0.952)
82.8	2.218	(0.117)	1.573	(0.165)
248.4	2.354	(0.184)	1.289	(0.155)

S.E.M. in parentheses

Shoot dry weights of mycorrhizal inoculated plants were greater than those of uninoculated plants at each of the three harvests for all the applied phosphorus levels (P varying from <0.05 to <0.001) (Fig. 3.2A, B, C.).

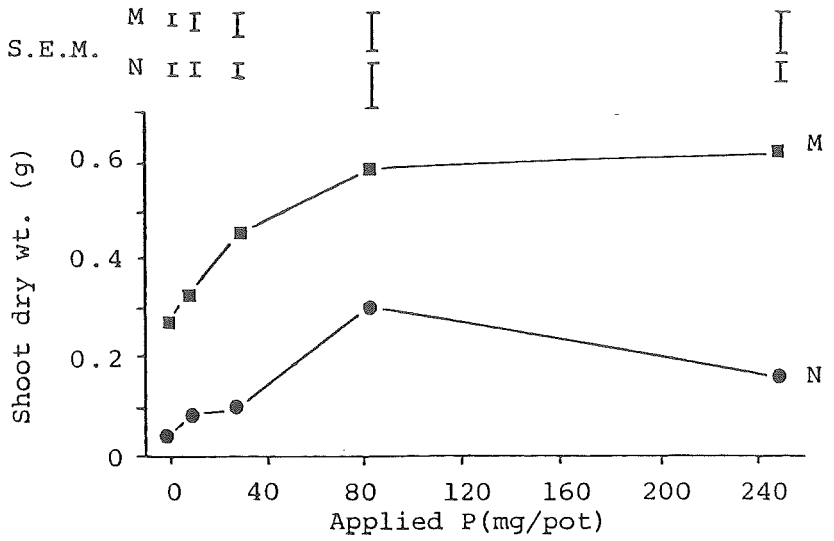
Figure 3.2 Effect of applied phosphorus and presence (M) or absence (N) of *G. fasciculatus* on shoot dry weight of white clover in Pawson Hill silt loam.

A. Harvest 1

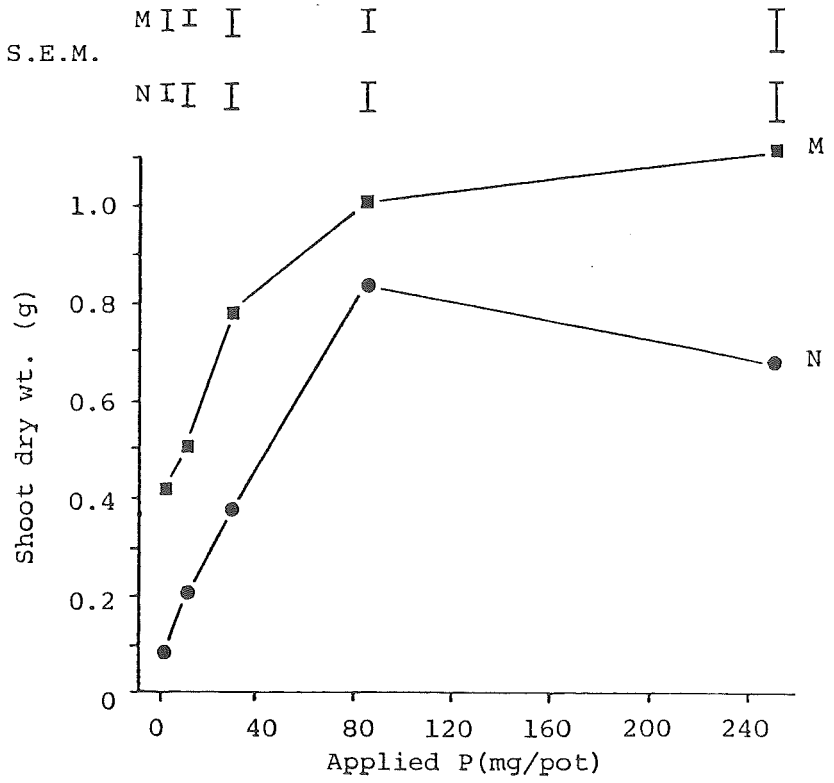
B. Harvest 2

C. Harvest 3

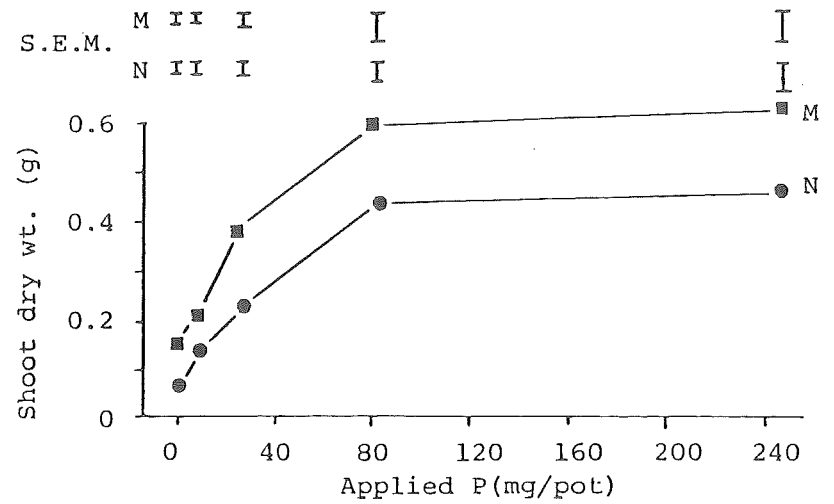
A. Harvest 1



B. Harvest 2



C. Harvest 3



Data for the magnitude of mycorrhizal response for the three harvests are presented in Table 3.7. There was a significant linear trend in the decrease in magnitude of mycorrhizal response with increase in phosphorus levels overall for harvests 1, 2 and 3 ($P < 0.001$). When the results for each individual harvest are analysed separately, a significant linear trend is obtained only for harvest 2 ($P < 0.001$).

Table 3.7 Magnitude of the mycorrhizal response (M/N) of white clover at the three harvests for Pawson Hill silt loam.

	*M/N		
Applied P (mg/pot)	Harvest 1	Harvest 2	Harvest 3
0.0	6.07 (1.06)	5.02 (1.06)	2.41 (0.48)
9.2	3.83 (0.88)	2.43 (0.57)	1.51 (0.24)
27.6	4.54 (1.09)	2.05 (0.34)	1.66 (0.19)
82.8	1.93 (0.73)	1.25 (0.13)	1.37 (0.17)
248.4	3.66 (0.86)	1.66 (0.26)	1.38 (0.23)

S.E.M. in parentheses

*Magnitude of mycorrhizal response determined as in Table 3.3

Plants inoculated and uninoculated with *G. fasciculatus* showed a significant linear component in response to applied phosphorus at each of the three harvests (P varying from <0.01 to <0.001). The decrease in shoot dry weights of uninoculated plants between P = 82.8 mg/pot and P = 248.4 mg/pot was not significant at both harvests 1 and 2.

The percentage of roots infected with *G. fasciculatus* determined at the third harvest decreased with increase in

phosphate levels (Table 3.8). Percentage mycorrhizal root infection at 9.2 mg P/pot was significantly less than at 0 mg P/pot. Differences in percentage root infection between 9.2 mg P/pot and 27.6 mg P/pot, 27.6 mg P/pot and 82.8 mg P/pot were not significant. Percentage root infection at 248.4 mg P/pot was significantly less than those at P = 82.8 mg/pot and below.

Table 3.8 Percentage of roots infected by *G. fasciculatus* at harvest 3 in Pawson Hill silt loam.

Applied P (mg/pot)	Mycorrhizal Root Infection (%)
0.0	45.7
9.2	33.4
27.6	31.4
82.8	34.2
248.4	22.1

S.E.M. = 3.99

L.S.D. (5%) = 11.3

3.3.3 Anatomical characteristics of *G. fasciculatus* infection (Experiments 1 and 2).

The anatomical characteristics of mycorrhizal roots at the different soil phosphorus levels in both experiments appeared normal and similar to the observations made in Chapter 2. Within each phosphorus treatment, both arbuscules and vesicles were present but they varied in abundance in the different root segments.

A small proportion of the root segments from the highest phosphorus levels used in the two soils produced atypical

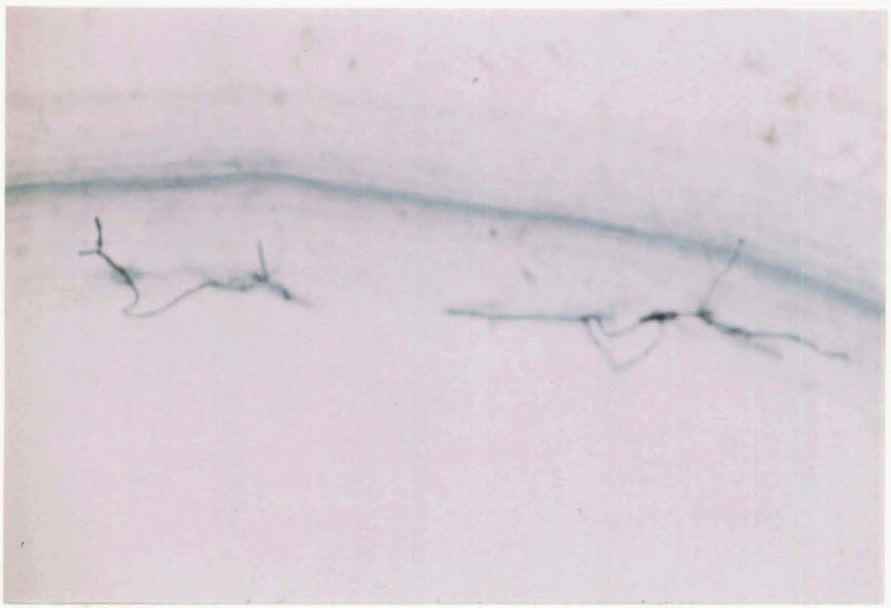
Plate 3.1 Abnormal structures of *G. fasciculatus* produced in roots of white clover grown in Tasman silt loam applied with 459.6 mg P/pot. Roots cleared in KOH and stained with lactophenol trypan blue.

A. Atypical infection units each consisting of an appressorium and a few thin hyphal branches on one side of the root segment. The stele of the root stained more darkly than the cortex cells. X330.

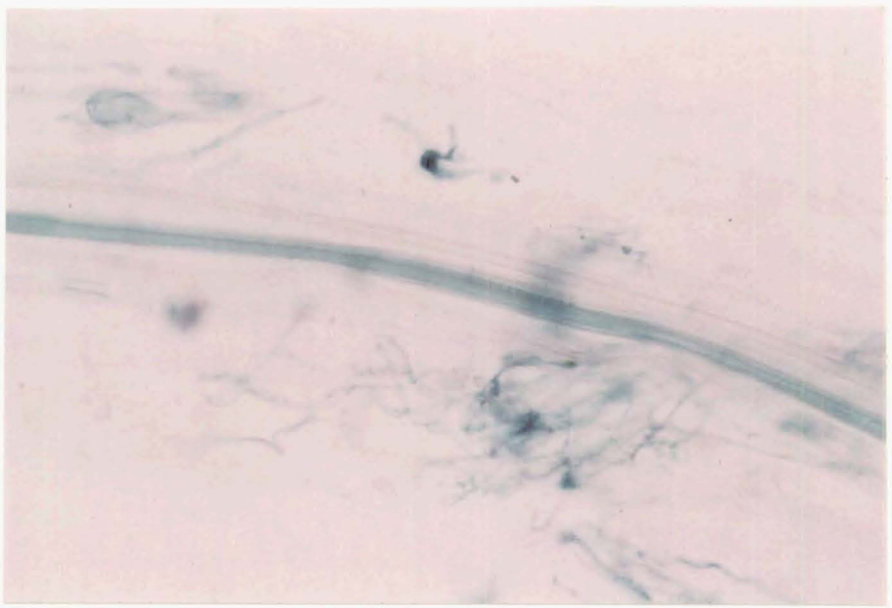
B. Small arbuscules and vesicles and lightly stained hyphal strands within the atypical infection units produced on white clover roots. X330.

Plate 3.1

A.



B.



structures. They appeared as infection units consisting of an appressorium with a few thin hyphal branches which spread to adjacent epidermal and cortical cells (Plate 3.1A). Small arbuscules or vesicles were occasionally observed in other infection units (Plate 3.1B). These infection units were normally scattered over the entire root segments. The degree of penetration and development of hyphae in these roots was, however, limited.

3.4 DISCUSSION

Growth of white clover was stimulated by application of phosphorus and inoculation with *G. fasciculatus* in both Tasman and Pawson Hill silt loams. Little or no growth was obtained in Tasman silt loam at 0, 9.2 or 27.6 mg P/pot for uninoculated plants. Growth appeared to be very dependent on mycorrhizal infection or application of high levels of phosphorus. There was a seven-fold increase in total shoot yield in the mycorrhizal treatment in Tasman silt loam compared to a four-fold increase for Pawson Hill at 0 mg P/pot. The total shoot yields of mycorrhizal inoculated or uninoculated plants in both soils were, however, not directly comparable because of differences in phosphate and harvesting treatments.

The results of this study agree with the findings of other workers on growth responses of white clover to mycorrhizal inoculation and phosphorus application in deficient soils (Crush, 1974; Hall *et al.*, 1977; Hall, 1978; Powell, 1980). In some of these studies, growth stimulation occurred only at low levels of applied phosphorus, with growth depression at higher phosphorus levels (Crush & Caradus, 1980; Hall *et al.*,

1977; Hall, 1978). In this study, significantly greater shoot yields were obtained for mycorrhizal plants at all levels of phosphorus in both soils. The magnitude of the mycorrhizal response, however, generally reduced as the soil phosphorus levels increased and with successive harvests.

The possible causes for persistent growth depressions of mycorrhizal plants as suggested by various workers are outlined in the general introduction (Chapter 1). According to Mosse (1973b), growth depression of onion plants was caused by toxic concentrations of phosphorus in the plant tissues. In this study, the highest phosphorus concentrations in the mycorrhizal and non-mycorrhizal shoot tissues from both soils (Appendix 5) were all within the 'safe excess' levels of white clover shoot phosphorus concentrations given by McNaught (1969), which might explain the absence of growth depression. Hall *et al.* (1977), however, found no evidence in his data with white clover plants that supports Mosse's (1973b) finding that growth depression was due to toxic concentrations of phosphorus in the plant tissues. The results of Abbott and Robson's experiment (1977), together with the above findings suggest that the relationship between yield and shoot phosphorus concentration as affected by mycorrhizal infection appears to be complex and to vary considerably with soil type.

Differences in mycorrhizal dependence of white clover in different soils has also been explained in terms of the initial soil phosphorus levels and phosphate retention. Powell (1980) found that white clover growth responded significantly to *Gigaspora margarita* and *Glomus tenuis* up to 60 mg P/pot (53 kg P/ha) and 200 mg P/pot (177 kg P/ha) in the Dunmore soil which he used - with initial $NaHCO_3$ extractable phosphorus of

9 µg/mℓ and phosphate retention of 97%. He commented that the much lower mycorrhizal dependence of white clover in Hall's experiment (1978) was probably due to the high fertility (16 µg/mℓ extractable phosphorus) and low phosphate retention (15%) of the Warepa soil he used. Crush and Caradus (1980), using two soils with high phosphate retention and very low initial available phosphorus (values not given), also noted a very low dependence of white clover on mycorrhizae with growth responses only in the lowest phosphate treatments. In the Tasman and Pawson Hill silt loams used in this study, the initial extractable phosphorus and phosphate retention was 7 µg/mℓ and 28% (Table 3.1), 18 µg/mℓ and 21% (Table 3.6) respectively. Growth of white clover appeared to be greatly dependent on mycorrhizal infection, with significant growth responses occurring up to 459.6 mg/pot (150.1 kg/ha) and 248.4 mg/pot (81.0 kg/ha) respectively of applied phosphorus. Hence, it appears that a number of factors in addition to initial phosphorus and phosphate retention values, determine the dependence of white clover on mycorrhizae in different soils. One factor which could explain the large growth responses of mycorrhizal plants at the earlier harvests in this study was that phosphorus was added to the plants after inoculation with *G. fasciculatus*, which might confer on inoculated plants a time advantage over the uninoculated, in their uptake of soil phosphorus and growth.

The percentage of mycorrhizal root infection was found to decrease with increase in phosphorus application for both Tasman and Pawson Hill silt loams. This confirms the findings of some earlier work by Daft and Nicolson (1969), Khan (1972) and Mosse (1973b). The mechanism responsible for inhibition of VA mycorrhiza formation under high soil phosphorus was

suggested by Ratnayake *et al.* (1978) to be caused by a membrane mediated decrease in root exudation. Soluble carbohydrate concentration has also been implicated in the phenomenon of inhibition of mycorrhizae formation at high phosphorus levels. Jasper *et al.* (1979) found that the effects of phosphorus supply on frequency of penetration by hyphae and subsequent mycorrhiza development closely paralleled effects of phosphorus supply on soluble carbohydate concentrations in roots. Graham *et al.* (1981), however, confirmed that the increase in VA mycorrhizal infection at low phosphorus status was due to the increase in membrane-mediated root exudation rather than to higher concentrations of reducing sugars or amino acids in roots, thus supporting the mechanism proposed by Ratnayake *et al.* (1978).

The anatomical characteristics of *G. fasciculatus* in white clover appeared to be unaffected by the different levels of phosphorus in the two soils except at the highest phosphorus levels. This was similar to the finding of Abbott and Robson (1978) working with subterranean clover. In another quantitative study, Abbott and Robson (1979) concluded that the anatomical characteristics of a *Glomus* species of subterranean clover were unaffected by different phosphorus supply, except for the elimination of vesicle formation at phosphate added above that required for maximum plant yield. Mosse (1973b), however, observed a great alteration in the anatomy of VA mycorrhizae in onion roots given large amounts of phosphate. The atypical structures observed in this study (Plate 3.1A & B) in roots from soils with the highest phosphorus were very similar to the abnormal VA infection in subepidermal cells of onion roots noted by Mosse (1973b - Plate 2, Figs 4 & 5).

As in her experiment, these structures probably formed as a result of the higher phosphorus concentration in the plant tissues.

CHAPTER 4

ROOT PATHOGEN STUDIES

4.1 INTRODUCTION

White clover is an important perennial pasture legume grown in many parts of the world. In permanent mixed pastures where its poor persistence has been a problem, the major cause was suggested to be rotting of roots by fungal pathogens, especially various *Fusarium* spp. and *Codinaea fertilis* (Leath *et al.*, 1971; Menzies, 1973a, b; Moody *et al.*, 1967).

Fusarium species are world-wide in distribution in cultivated and non-cultivated soils (Booth, 1971) and are fungi commonly associated with roots of white clover and other forage legumes. These include species like *F. oxysporum*, *F. solani*, *F. avenaceum* and *F. culmorum* (Burgess *et al.*, 1973; Fezer, 1961; Moody *et al.*, 1967; Sundheim, 1970). Of these, *F. oxysporum* is known to cause serious wilt and rot diseases of many economically important crop plants (Booth, 1971). *F. avenaceum*, equally ubiquitous, is known to cause root rots of wheat, rye, maize, legumes and conifers and damping-off of nursery seedlings. In particular, it has been shown to be associated with root rot and poor persistence of subterranean clover (Burgess *et al.*, 1973; Kellock *et al.*, 1978) and red clover (Sundheim, 1970). Both *F. oxysporum* and *F. avenaceum* have been consistently isolated from roots of white clover grown in various New Zealand pastures (Skipp, pers. comm.; Thornton, 1965).

Codinaea fertilis Hughes & Kendricks was first reported to be the primary pathogen of white clover roots in New Zealand by Menzies (1973a). Prior to that, it had only been reported growing on dead leaf tissues of deciduous trees (Ellis, 1976). Campbell (1980) also showed *C. fertilis* to be the main cause of Ladino clover root rot in North Carolina, U.S.A. More recently in glasshouse studies, he found *C. fertilis* pathogenic on roots of a whole range of clovers including Persian, arrow leaf, subterranean, red, crimson, white, alsike, white sweet and hop clover, hairy vetch, alfalfa, Korean lespedeza, Kobe Striate lespedeza, corn, snap bean, soybean and garden pea (Campbell, 1982).

Thielaviopsis basicola (Berk. & Br.) Ferraris, a common soil-borne fungus and the causal agent of root rot of tobacco *(Nicotiana tabacum)* and bean *(Phaseolus vulgaris)*, was among the fungi found associated with white clover roots by Kilpatrick (1959) in U.S.A. and Skipp and Christensen (pers. comm.) in New Zealand. Its role as a root pathogen of white clover has, however, not been firmly established.

Apart from the reports by Menzies (1973a) in which *C. fertilis* was shown as the primary pathogen of white clover and by Thornton (1965) where *F. avenaceum* was shown pathogenic under glasshouse conditions, the role of other commonly isolated fungi as pathogens has not been established.

In this study, five fungal isolates from white clover roots were tested for pathogenicity on white clover seedlings under glasshouse conditions. Infection and colonization of these pathogens was followed microscopically. The characteristic features of the pathogenic fungi aided in differentiating

between mycorrhiza and pathogens in later work.

4.2 MATERIAL AND METHODS

4.2.1 Fungal isolates

Five fungal species isolated from white clover roots were used in this study. They were:

		Source of Isolates
(1)	*Codinaea fertilis*	Kaikohe
(2)	*Fusarium avenaceum* (Fr.) Sacc.	Woodville
(3)	*Fusarium oxysporum* Schlecht. isolate 1.	Woodville
*(4)	*Fusarium oxysporum* Schlecht. isolate 2.	Invermay
(5)	*Thielaviopsis basicola* (Berk. & Br.) Ferraris	North Auckland

* Isolate kindly supplied by Dr. I. R. Hall (Invermay Agricultural Research Centre, Mosgiel, New Zealand). The other 4 isolates kindly supplied by Dr. R. Skipp, (D.S.I.R., Palmerston North, New Zealand).

4.2.2 Maintenance of fungal cultures

Cultures of fungal isolates were maintained on slopes of the following agar media in McCartney bottles.

Potato dextrose agar (PDA)	-	*C. fertilis*
Potato sucrose agar (PSA)	-	*F. avenaceum*
		F. oxysporum isolate 1
		F. oxysporum isolate 2
V_8 juice agar (V_8A)	-	*T. basicola*

The procedure for the preparation of these agar media is outlined in Appendix 6.

4.2.3 Soils

Both Tasman and Pawson Hill silt loams were used. The preparation of Tasman and Pawson Hill silt loams is as outlined in Chapters 2 and 3 respectively. The soil mixture was weighed and potted into each of the 10 cm 'Squat pots' as described previously and basal nutrients added.

4.2.4 Fungal inocula

Inocula of the fungal isolates consisted of cultures grown in a mixture of ground oatmeal and sand. They were prepared by adding 3 agar discs of each fungus to a sterilized medium consisting of 200 ml each of ground oatmeal and sand and 60 ml of distilled water in a one litre Erlenmeyer flask. Cultures of *C. fertilis* and *T. basicola* were incubated in darkness at 25.5-27.5^{o}C for 21 days. Those of *Fusarium* species were incubated in 12 h daylight at 22-25^{o}C for 10 days. Each flask was shaken daily to disperse the fungus throughout the medium.

4.2.5 Inoculation of host plants

The preparation of white clover seeds for germination was as described in Chapter 2. The seeds were left to germinate on 2% water agar at 25^{o}C for 48 hours before being transplanted.

Ground oatmeal sand culture (25 ml) of each fungus was mixed with the soil in each pot and topped with unmixed fumigated soil 1.5 cm in thickness. Three germinated seedlings were planted at the centre of each pot. Rhizobial suspension (1 ml) was pipetted onto the soil around the seedlings. Control pots were mixed with an equal volume of autoclaved ground oatmeal culture of each fungus. The pots were placed in a glasshouse in which the air temperature fluctuated between 10^{o}C and 30^{o}C and watered with distilled water.

4.2.6 Harvesting and disease assessment

One seedling from each of the five pots in each treatment was harvested 7, 14 and 21 days after inoculation. The roots were carefully washed free of soil and examined for root disease symptoms under a stereomicroscope.

Root segments with disease symptoms were randomly selected from seedlings harvested 21 days after inoculation for reisolation studies. They were surface-sterilized with 0.5% sodium hypochlorite solution for 2 min, rinsed three times with sterile distilled water, dried with sterile filter paper, cut into 2-3 mm segments and plated onto acidified PDA (Appendix 6) for all the fungal isolates. The plates were incubated at 25^{o}C and colonies grown were subcultured onto fresh non-acidified agar media. The mycelia and spores of the pathogenic fungi were stained with lactophenol cotton blue (Appendix 7), mounted in lactophenol and examined microscopically.

4.2.7 Infection and colonization of host roots

Root samples of seedlings harvested at days 14 and 21 and showing symptoms of root diseases were processed for microscopic examination. One method involved clearing and staining whole root segments with lactophenol trypan blue as outlined in Appendix 1. Excess stain was removed with lactophenol and roots mounted on microscope slides. In the second method used, 0.5 cm long root segments were fixed in 3% glutaraldehyde made up in a 0.1 M sodium phosphate buffer at pH 6.8, dehydrated and embedded according to the procedure outlined in Appendix 2. The blocks were sectioned (10-15 μm thick) using a rotary microtome. Thin sections were mounted onto slides and stained with safranin-fast green (Appendix 3) or periodic acid-Schiff's stain (Appendix 4).

The infection and colonization of the host roots was also followed using inoculated seedlings grown on water agar. White clover seeds were surface-sterilized and germinated as described previously and transferred aseptically to 2% water agar plates. A spore and mycelial suspension of the pathogenic isolates of *C. fertilis*, *F. avenaceum* and *T. basicola* was transferred by a flamed loop onto the seedling roots. The plates were incubated in 12 h daylight at 25°C. Randomly selected root segments of three seedlings per treatment were fixed and processed for microscopic examination as described above at 1, 3, 5, 7, 14 days after inoculation.

4.3 RESULTS

4.3.1 Pathogenicity tests

Of the five fungal isolates tested for pathogenicity, only *C. fertilis*, *F. avenaceum* and *T. basicola* produced necrotic root lesions and were consistently reisolated from the inoculated roots of seedlings grown in both Pawson Hill and Tasman silt loams. They were thus considered as pathogenic to white clover seedling roots under the conditions of the experiment. The two isolates of *F. oxysporum* produced very little damage on the seedling roots and were reisolated from a very small proportion of the root segments plated.

The cultural characteristics of these pathogenic fungi are given below.

(a) *C. fertilis*

Colonies of *C. fertilis* on PDA are slow growing. They initially appear white, turning grey with age. Submerged hyphae on the reverse side of the plate show dark pigmentation at the centre, but white at the periphery (Plate 4.1A). Aerial hyphae generally aggregate into thread or rope-like strands. The conidiophores are stalked polyphialides which are straight or gently curved, unbranched and septate (Plate 4.2A). They arise singly or in groups of 3 to 4 from knots of hyphal cells. A funnel-shaped collarette bearing phialospores is usually observed at the tip of the conidiophore and remains of other collarettes observed on the sides (Plate 4.2A). Phialospores are curved, non-septate, slightly tapered at one end and more rounded at the other end and usually with a setula on both ends (Plate 4.2B).

(b) *F. avenaceum*

Colonies of *F. avenaceum* growing on PSA are very floccose, especially when incubated in complete darkness. The colonies are pink fringed with white, becoming yellowish-brown in the centre. Submerged hyphae appear deep red fringed with white on the reverse side of the dish (Plate 4.1B). For colonies incubated in 12 h daylight, masses of orange sporodochia are produced in concentric zones (Plate 4.1C).

Two types of conidia are produced by *F. avenaceum* in culture under both light and dark conditions. The first type, produced from polyblastic cells, are fusoid (10-30 x 3.5-4.5 µm) and 1-3 septate (Plate 4.2C). They are not produced in abundance and hence not as commonly observed as the orange coloured macroconidia produced within sporodochia (Plate 4.2D). Macroconidia are also fusoid but narrower and longer (50-75 x 3.5-4 µm), with 4-7 septa and are curved, with an elongated apical cell and a well marked foot cell (Plate 4.2E).

(c) *T. basicola*

Colonies of *T. basicola* on V_8A are greyish black in colour, becoming darker with age in the centre (Plate 4.1D). Two types of unicellular conidia are produced: macroconidia (chlamydospores) produced in short chains from hyaline basal cells and microconidia (endoconidia) produced within endoconidiophores. Each chlamydospore is subrectangular in shape and slightly rounded at the tips (Plate 4.2F). Endoconidia are cylindrical, truncate at the ends and are liberated through the apex in succession (Plate 4.2G).

Plate 4.1 Colony characteristics of *C. fertilis*, *F. avenaceum* and *T. basicola* on agar media.

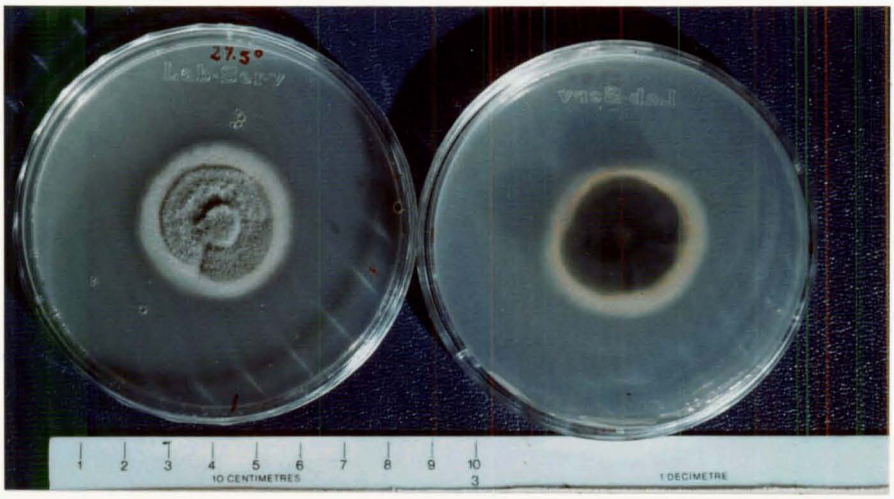
A. Eight-day-old colonies of *C. fertilis* on PDA incubated at 27.5°C in darkness.

B. Colonies of *F. avenaceum* on PSA incubated at 25°C for four days in darkness.

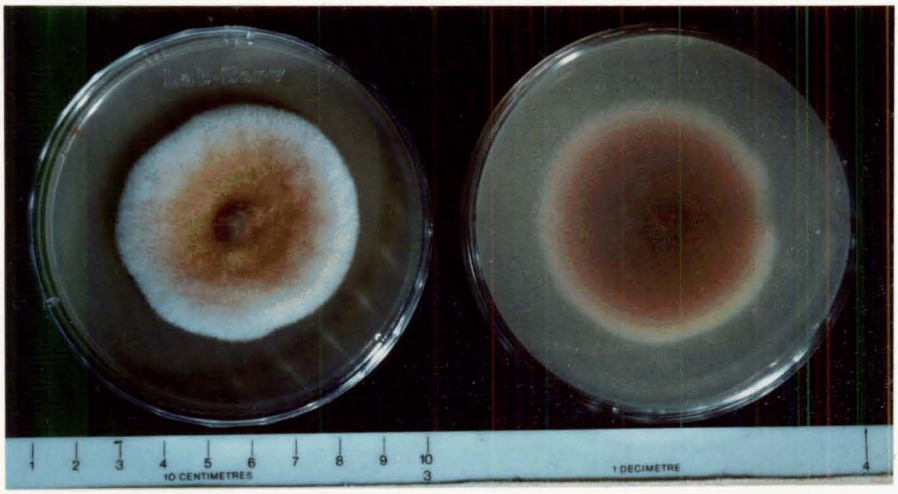
C. A colony of *F. avenaceum* on PSA incubated for seven days in 12 h daylight at 22-25°C.

D. Colonies of *T. basicola* on V_8A incubated at 25.5°C in darkness for eight days.

Plate 4.1



A.



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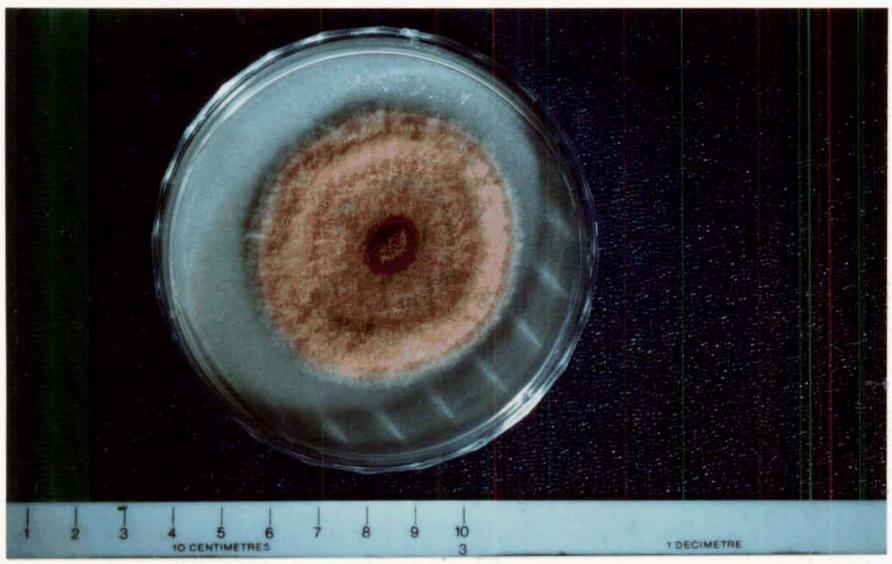

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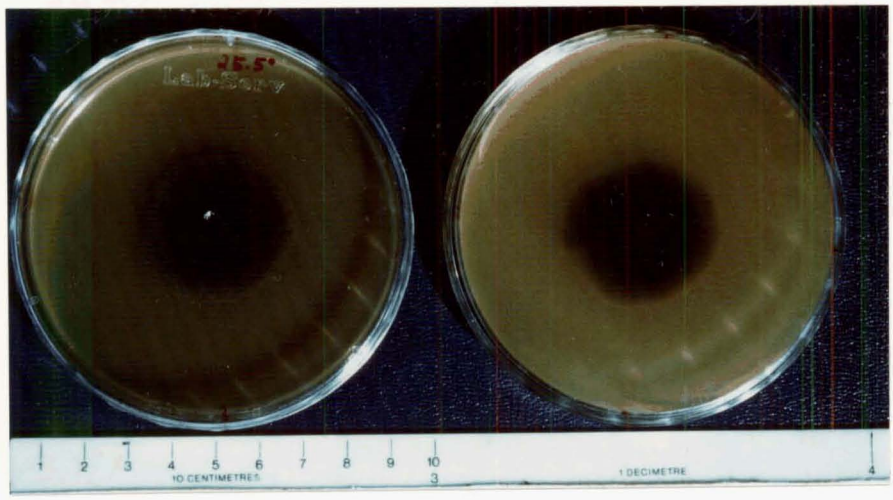


D.

Plate 4.2 Reproductive structures of *C. fertilis*, *F. avenaceum* and *T. basicola* produced on agar media.
Structures stained with lactophenol cotton blue and mounted in lactophenol on microscopic slides.

A. Septate conidiophores of *C. fertilis* bearing funnel-shaped collarettes (arrows) and phialospores (curved arrows). X1,700.

B. Phialospores of *C. fertilis* bearing a setula (arrows) at each end. X1,300.

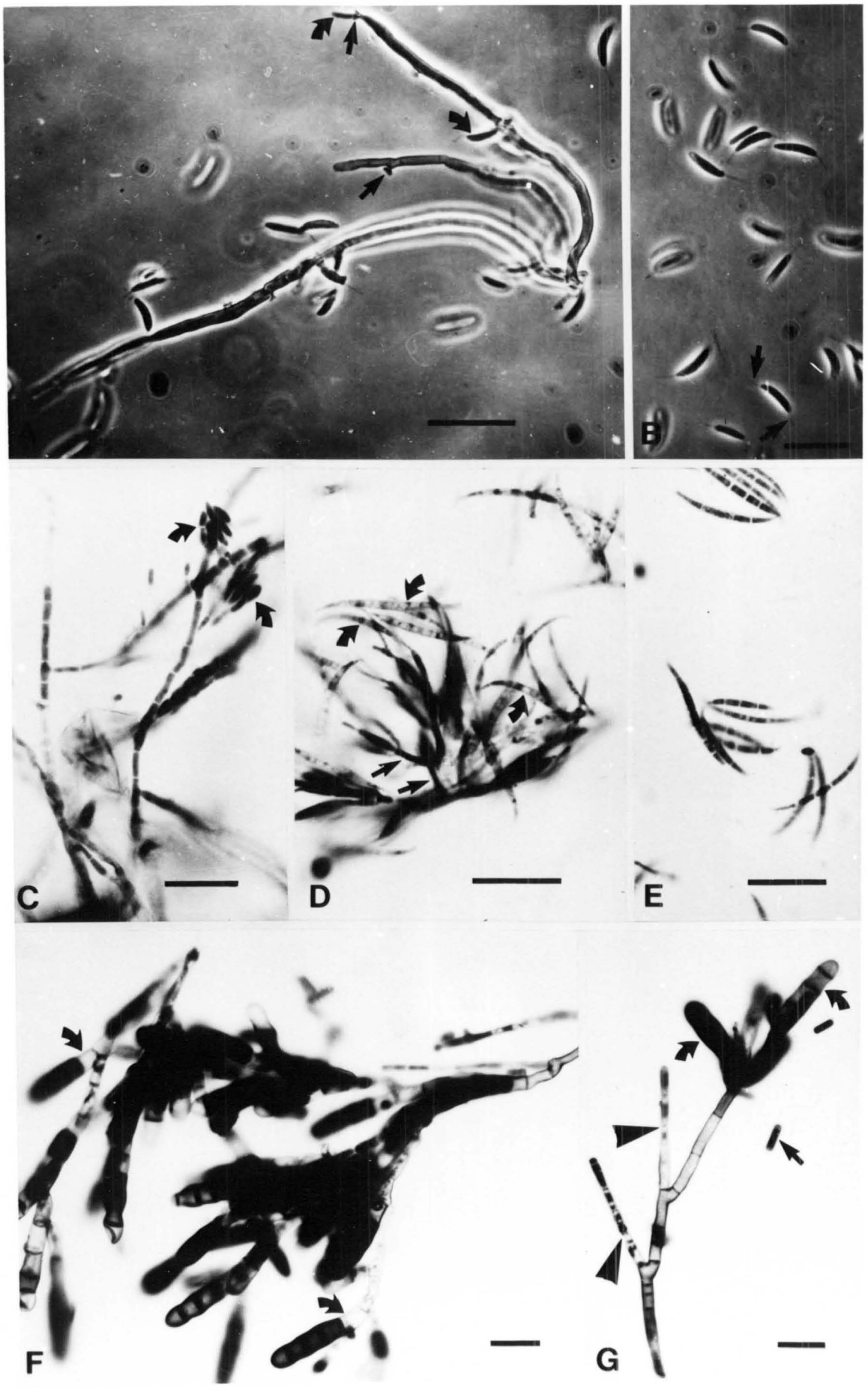
C. Conidia (curved arrows) of *F. avenaceum* produced from polyblastic cells of hyphae. X1,400.

D. Macroconidia (curved arrows) of *F. avenaceum* arising from short phialides (arrows) within a sporodochium. X1,700.

E. Macroconidia of *F. avenaceum* containing different numbers of septa. X1,500.

F. Masses of chlamydospores of *T. basicola* produced in short chains by the hyaline basal cells (curved arrows). X950.

G. Hyphal branch of *T. basicola* bearing short chains of chlamydospores (curved arrows), and endoconidiophores (arrowheads) which bud off endoconidia (arrow). X900.


B
C
D
E
F
G

4.3.2 Symptoms of infection

Small necrotic lesions first appear on seedling roots seven days after inoculation with *C. fertilis*. These develop into larger areas of dark brown discolouration or lesions over the tap and lateral roots within 14 days (Plate 4.3A). Infected root tips generally turn brown and further elongation of the roots is stopped. New lateral roots, however, continue to be produced from uninfected parts of the root system. On 21-day-old seedlings, symptoms range from small discrete brown lesions to general root necrosis that extends for a considerable length along the roots.

Seedlings inoculated with *F. avenaceum* show brown discolouration on the roots 14 days after inoculation. Darker brown lesions are observed on the crowns, tips of some tap and lateral roots, as well as on regions where the lateral roots emerge from the tap roots (Plate 4.3B). Newly emerging lateral roots that became infected at the tips remain as short brown stumps.

For seedlings inoculated with *T. basicola*, brown streaks appear on the roots seven days after inoculation. As infection develops, these brown streaks coalesce into larger areas of dark brown lesions or general root necrosis (Plate 4.3C). Dark brown to black chlamydospores are usually observed on the infected roots. They were produced either on the root surface or within the epidermal or outer cortical cells.

Plate 4.3 Root disease symptoms produced on 14-day-old seedlings by fungal pathogens.

A. Roots of seedlings infected with *C. fertilis* showing discrete brown lesions and general root necrosis. X4.0.

B. Typical root disease symptoms produced by *F. avenaceum* infection. Note the general brown discoloration and the darker brown lesion on the lateral root. X10.0.

C. Roots of seedlings infected with *T. basicola* showing severe general root necrosis and a few dark brown lesions on the lateral roots. Note the blackish chlamydospores which are scattered over the roots. X4.0.

Plate 4.3

A.

B.

C.

4.3.3 Infection and colonization of host roots by pathogens

Descriptions of the colonization processes of the pathogens are based on observations of thin sections stained with safranin-fast green or periodic acid Schiff's stains. Root clearing and staining with the lactophenol trypan blue method proved unsatisfactory for this study. Hyphae of the pathogens were not intensely stained and could not be observed clearly.

4.3.3.1 *Codinaea fertilis*

Within 24 hours after inoculation, phialospores on the root surface germinate and hyphal fragments start to grow. These hyphae sometimes grow extensively on the root surface before penetrating the epidermal cells through the wall or their intercellular spaces (Plate 4.4A, B). In young roots (harvested less than seven days after inoculation), an extensive development of hyphae spreads from the epidermal to inner cortical layers. The hyphae, produced mainly within the cells, are fine and grow usually perpendicular to the length of the root. They swell before and after coming into contact with a host wall (Plate 4.4A).

Various responses to infection are observed in root cells infected with *C. fertilis*, seven days after inoculation. The walls and contents of infected cells are usually more deeply staining than uninfected cells (Plate 4.4C), and in some instances stain deep red with safranin-fast green (Plate 4.5A, B, C). Lignituber-like outgrowths, which also stain red, are commonly observed on the walls of epidermal and outer cortical cells (Plate 4.4C; 4.5A, C). These cells may contain sparse or apparently dead hyphae, but in many cases they are not pene-

Plate 4.4 Infection and colonization of host roots by *C. fertilis*. Sections stained with safranin-fast green.

A. Longitudinal section of root two days after inoculation with *C. fertilis*. Root tissues are extensively colonized by hyphae from the epidermis to the inner cortex. X1,300.

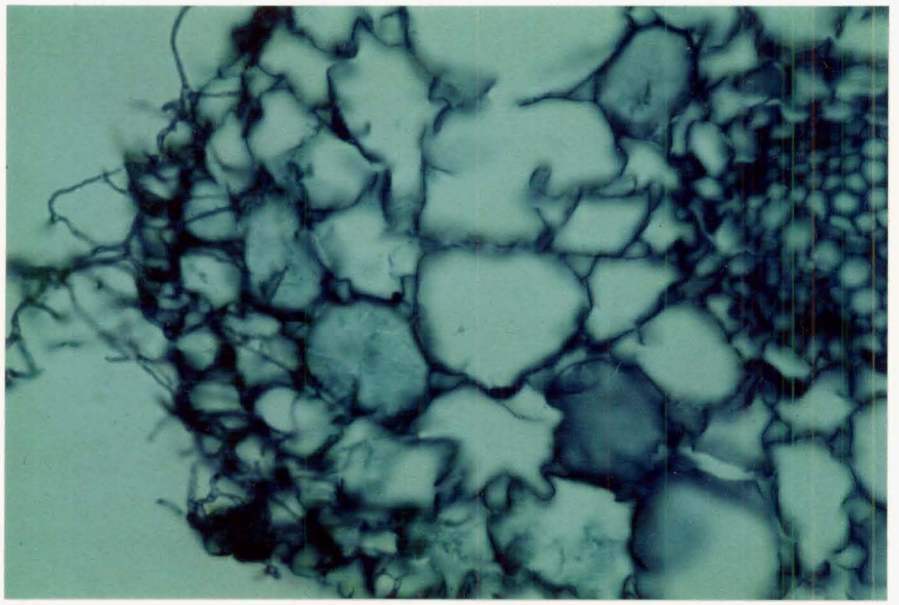
B. Transverse section of root three days after inoculation with *C. fertilis* showing growth of hyphae on the root surface, their penetration and growth in the outer cortical cells. X1,300.

C. Transverse section of root seven days after inoculation with *C. fertilis*. Note the lignituber-like outgrowths on the walls of some epidermal and outer cortical cells. Few hyphae are present in these cells. X1,300.

Plate 4.4

A.

B.



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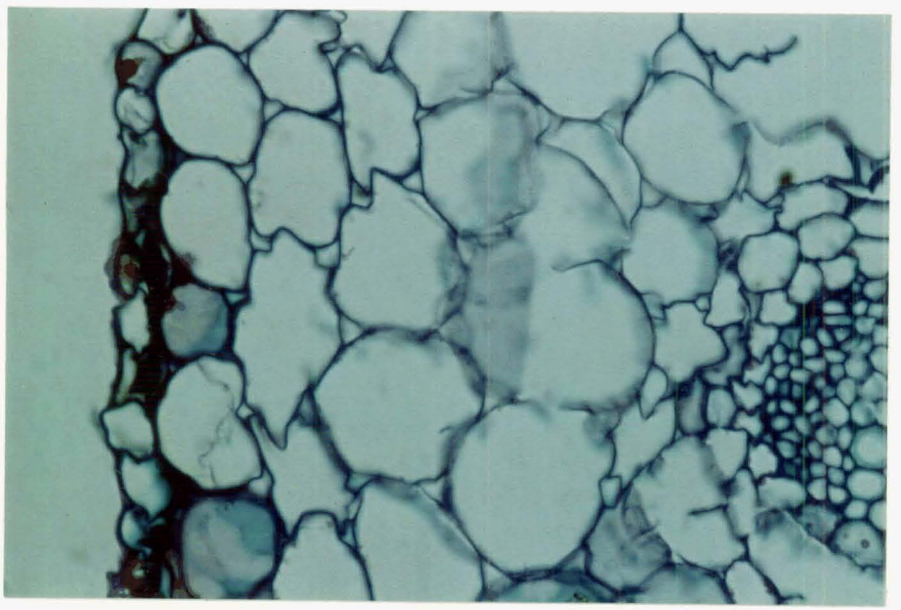


Plate 4.5 Section of roots showing features of host responses to infection by *C. fertilis*.
Roots were harvested more than seven days after inoculation with *C. fertilis*. Sections stained with safranin-fast green.

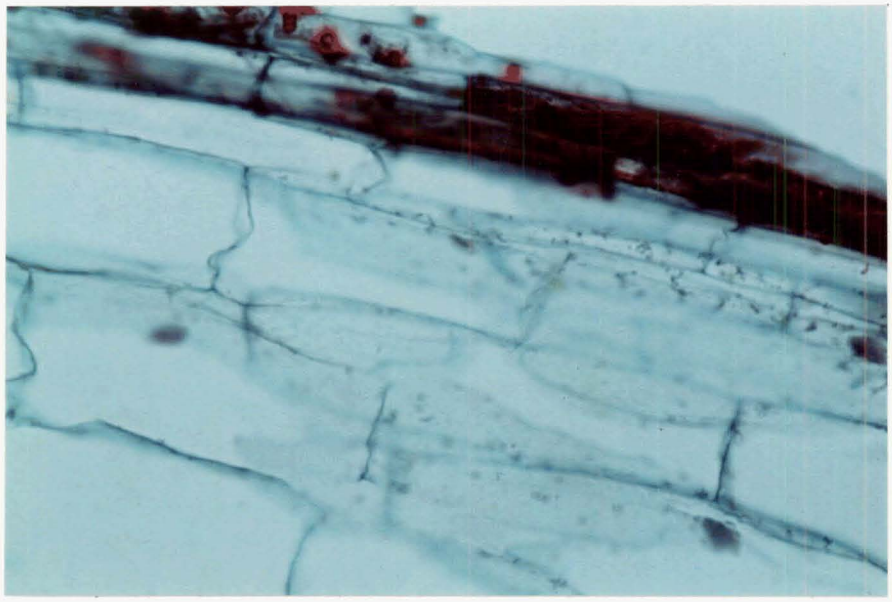
A. Longitudinal section of root with outer cell layers showing darkly stained walls and cell contents. Note the lignituber-like outgrowths arising from the walls of some cells. X1,300.

B. Oblique section of root with a lateral branch. The darkly stained cells correspond to necrotic areas observed visually. X825.

C. Part of the root as shown in B, at a higher magnification. Infected cells appear to contain sparse hyphae. The walls with the lignituber-like outgrowths stained red with safranin. X1,300.

Plate 4.5

A.



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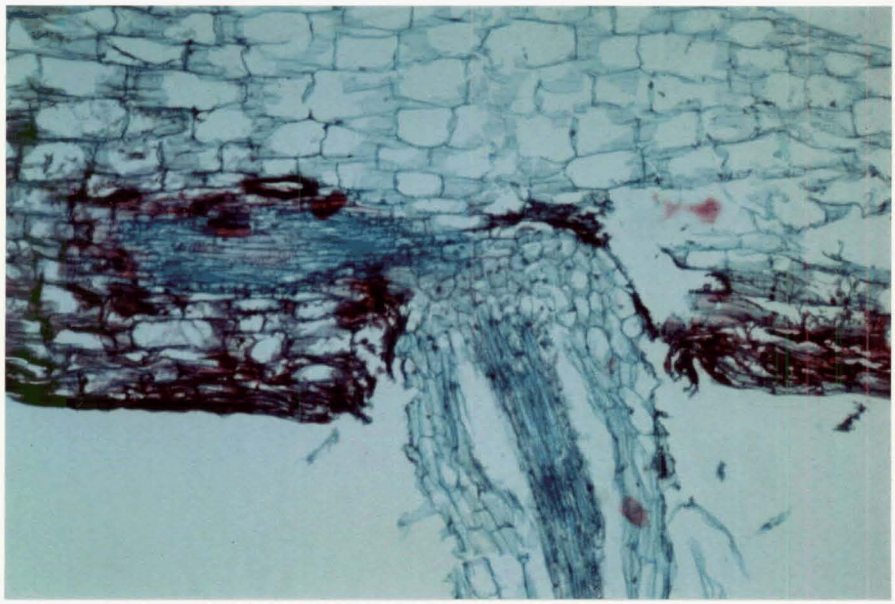

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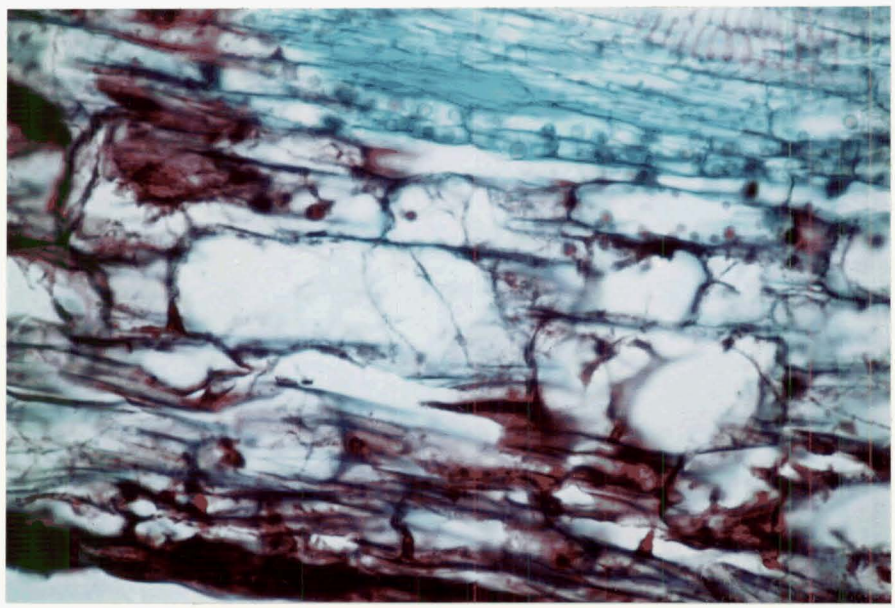


trated. In areas of roots where these host responses are observed, infection by *C. fertilis* appeared to be limited to the surface cell layers or to groups of cells in the cortex.

Microscopic examination of root sections from glasshouse grown plants two or three weeks after inoculation revealed similar features of *C. fertilis* infection and host responses to infection.

4.3.3.2 *Fusarium avenaceum*

Hyphae of *F. avenaceum* penetrate roots through the epidermal wall or intercellular spaces of the epidermis. Within the root, infection spreads through the cortical cell layers by long, coarse, septate hyphae (Plate 4.6A). Initially these hyphae branch and grow extensively in the intercellular spaces (Plate 4.6A) but become intracellular as infection spreads. Some cortical cells eventually become packed with hyphae (Plate 4.6B, C). Growth of hyphae in the xylem vessels may also occur (Plate 4.6C).

Various responses to infection were observed in host cells. Infected epidermal cells may lose their turgidity and collapse and the cytoplasmic contents and walls of infected epidermal and cortex cells become densely stained (Plate 4.7B). Infected xylem vessels become filled with a granular, densely stained material (Plate 4.6C). In some cases, compact stroma of hyphae extend from the epidermis to the outer cortex, but give no further growth into the inner cortex (Plate 4.7A).

Roots of seedlings in glasshouse pathogenicity tests, harvested at 14 and 21 days after inoculation, showed similar long, coarse, septate hyphae of *F. avenaceum* within the cortex cells or intercellular spaces. These hyphae, however developed extensively only in isolated groups of root cells which probably

Plate 4.6 Infection and colonization of host roots by *F. avenaceum*.
Sections stained with periodic acid-Schiff's stain.

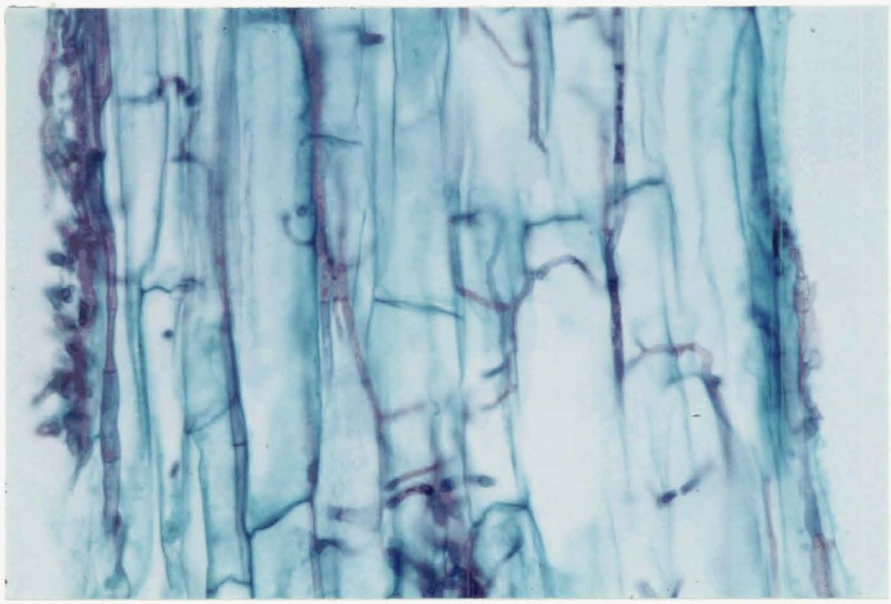
A. Longitudinal section of root showing long, coarse, septate hyphae of *F. avenaceum* on the root surface and also in the intercellular spaces of the cortex. X1,300.

B. Longitudinal section of root showing a few cortical cells completely filled with hyphae of *F. avenaceum*. X1,300.

C. Section showing extensive development of *F. avenaceum* hyphae that extends from the epidermis to the vascular tissues. Note the compact masses of hyphae in the intercellular spaces of the cortex and also within the xylem vessels. X1,300.

Plate 4.6

A.



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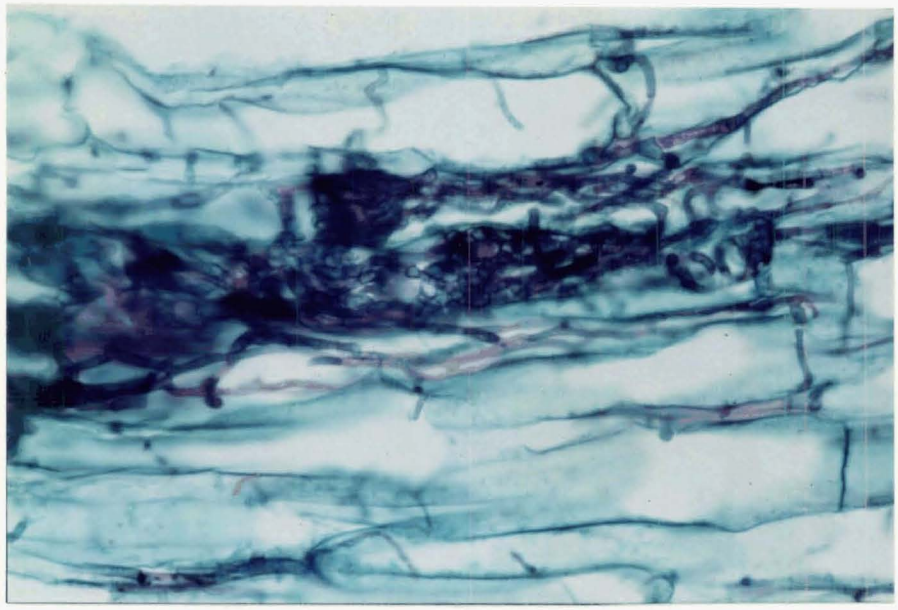


C.

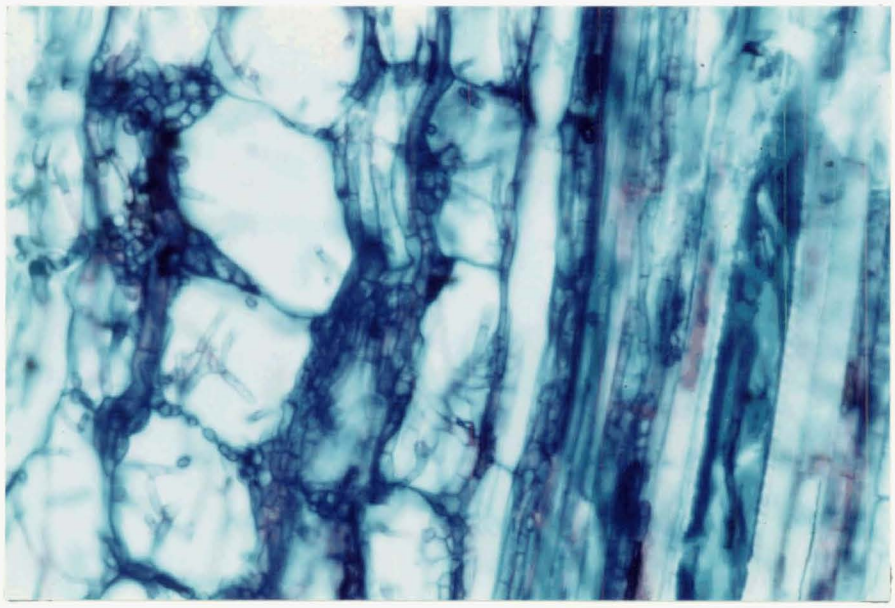


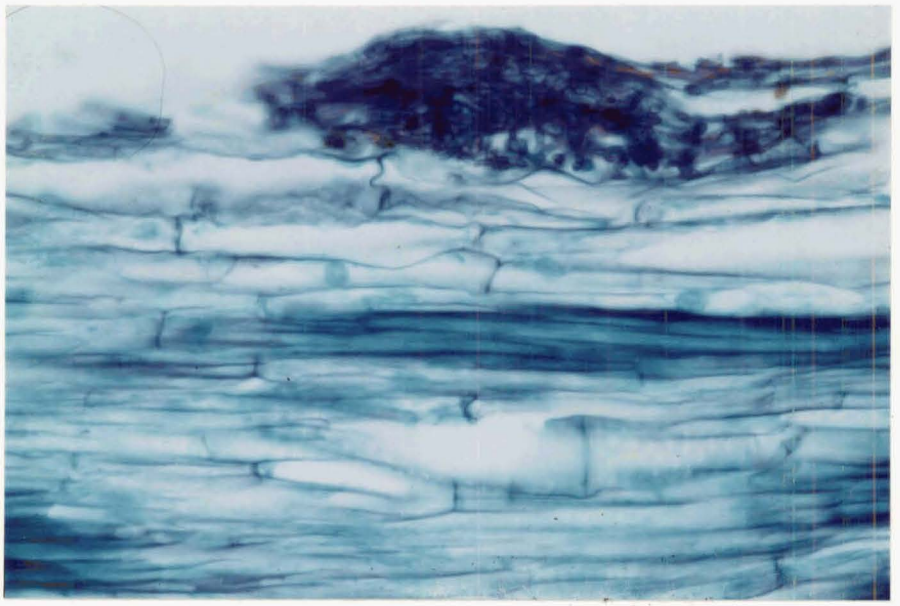
Plate 4.7 Infection features of *F. avenaceum* and host responses to infection. Roots cut longitudinally.

A. Section of root showing a compact stroma of *F. avenaceum* hyphae that extend from the epidermis to the outer cortex. Section stained with periodic acid-Schiff's stain. X1,300.

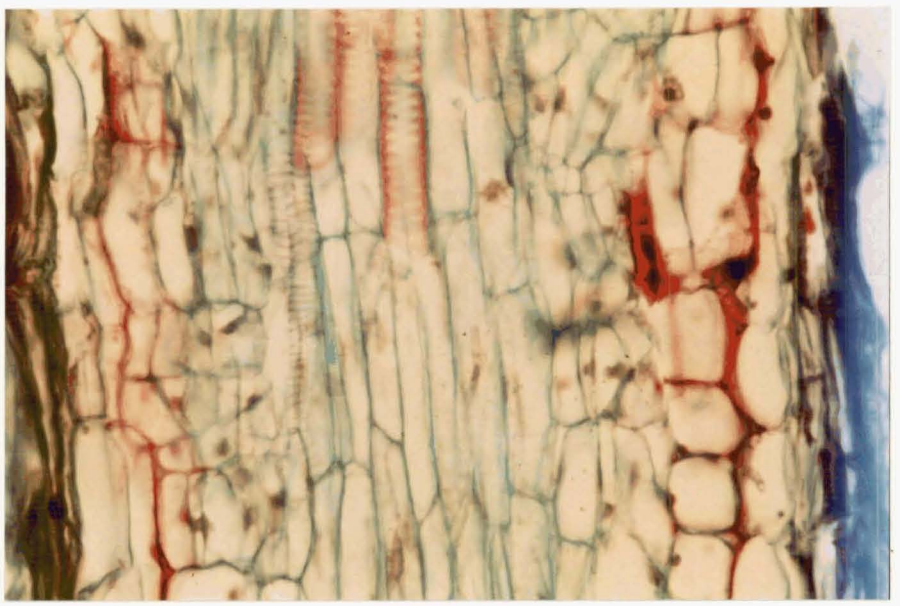
B. Section of root showing collapsed epidermal and outer cortex cells with darkly stained walls and cell contents, in response to infection by *F. avenaceum*. Section stained with safranin-fast green. X1,300.

Plate 4.7

A.



B.



correspond to the dark brown lesion areas.

4.3.3.3 *Thielaviopsis basicola*

Hyphae produced from the regrowth of hyphal fragments or germinated endoconidia and chlamydospores on the root surface penetrate epidermal cells directly without forming appressoria. Within the epidermal cells, the hyphae branch and develop into chains of swollen septate hyphae. These hyphae are constricted at each septum (Plate 4.8A). They eventually fill the entire volume of the cells. Infection pegs produced from branches of these hyphae invade adjacent cortex cells and develop into similar intracellular constricted hyphae. Other branches of the intracellular hyphae penetrate into the intercellular spaces to produce non-constricted septate hyphae. These grow along the longitudinal axis of the root and send out side branches which penetrate adjacent cortical cells (Plate 4.8B). These branches develop either into more intracellular constricted hyphae, or into hyphae which produce chlamydospores within the cortex cells (Plate 4.8C), or which grow further to the surface of the root and develop into chlamydospores or endoconidiophores which bud off endoconidia (Plate 4.9A).

In most of the roots examined, infection by *T. basicola* extends to small groups of cells in adjacent areas (Plates 4.8C, 4.9A, C). These groups of infected cells probably correspond to the dark brown lesions scattered over the root system. In a few advanced cases of infection, intracellular hyphae and chlamydospores were observed also in xylem vessels.

Cytoplasmic contents of infected cells become granular in appearance and are more darkly stained than in uninfected cells (Plate 4.8C). The walls of infected cells also appear to be more densely stained than those of uninfected cells (Plate 4.8C; 4.9B), with the walls of infected epidermal cells having collapsed in some cases (Plate 4.9B). Lignituber-like outgrowths were observed only occasionally on the wall of cells where development of *T. basicola* was limited or absent (Plate 4.9B, C).

Roots of seedlings inoculated with *T. basicola* and left to grow under glasshouse conditions showed similar features of infection and development of *T. basicola* within the root tissues.

4.4 DISCUSSION

In this study, *C. fertilis*, *F. avenaceum* and *T. basicola* are shown to be capable of invading and producing necrotic lesions on roots of white clover seedlings grown in Pawson Hill and Tasman silt loams under glasshouse conditions. The results thus confirmed the finding of Menzies (1973a). Campbell (1980; 1982) and Skipp and Christensen (1982) concerning the pathogenicity of *C. fertilis* on white clover roots under glasshouse conditions. The results of microscope studies revealed that discoloration or necrotic lesions on the roots are associated with extensive development of hyphae within the root tissues, or with limited spread within the first few cortical layers. The production of lignituber-like outgrowths was a consistent feature associated with limited spread of the hyphae or absence of penetration of the host tissues, and appears to be a host

Plate 4.8 Infection and colonization of host roots by *T. basicola*.

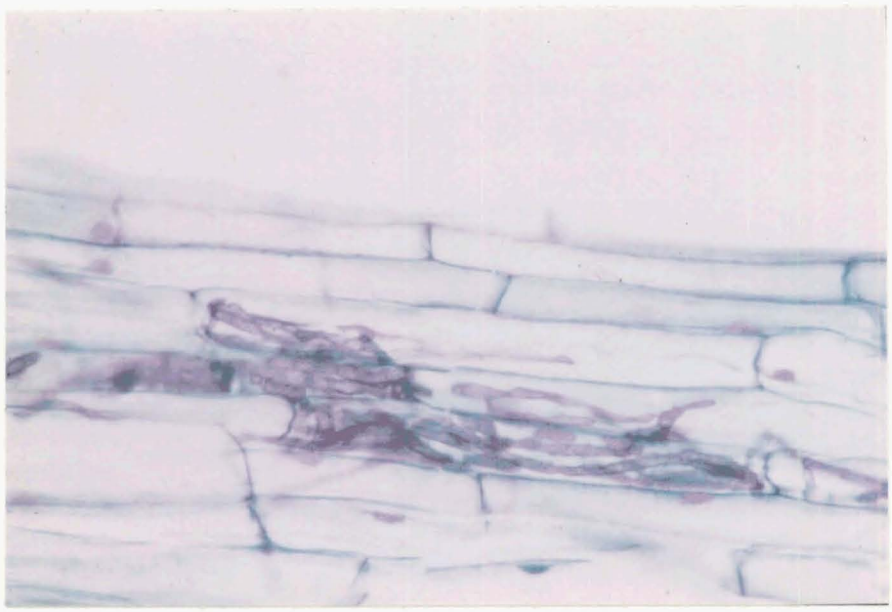
A. Section through the root epidermis showing intracellular constricted hyphae of *T. basicola* in a few cells.
Section stained with periodic acid-Schiff's stain. X1,300.

B. Section of root through the cortex showing the development of intercellular hyphae and their side branches.
Section stained with safranin-fast green.
X1,300.

C. Cortex cells containing intracellular constricted hyphae and chlamydospores. The walls and cytoplasmic contents of infected cells are more darkly stained than those of uninfected cells. Note the granular appearance of the cytoplasm of infected cells. X1,300.

Plate 4.8

A.



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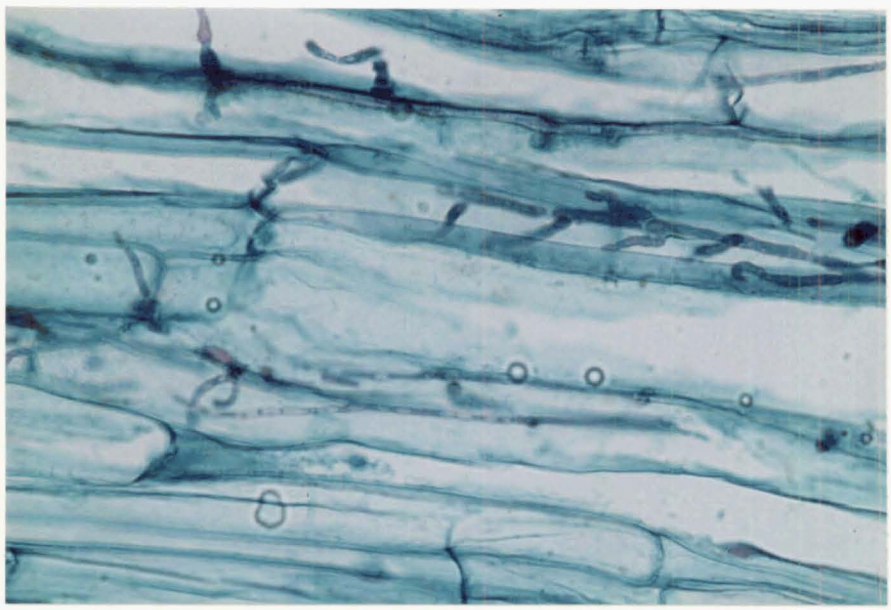


C.

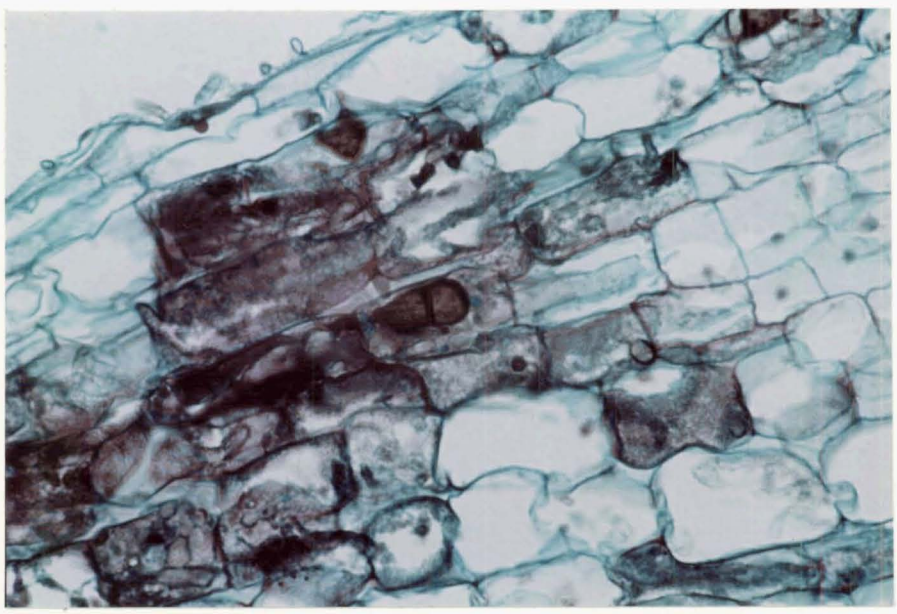


Plate 4.9 Infection features of *T. basicola* and host responses to infection.
Sections stained with safranin-fast green.

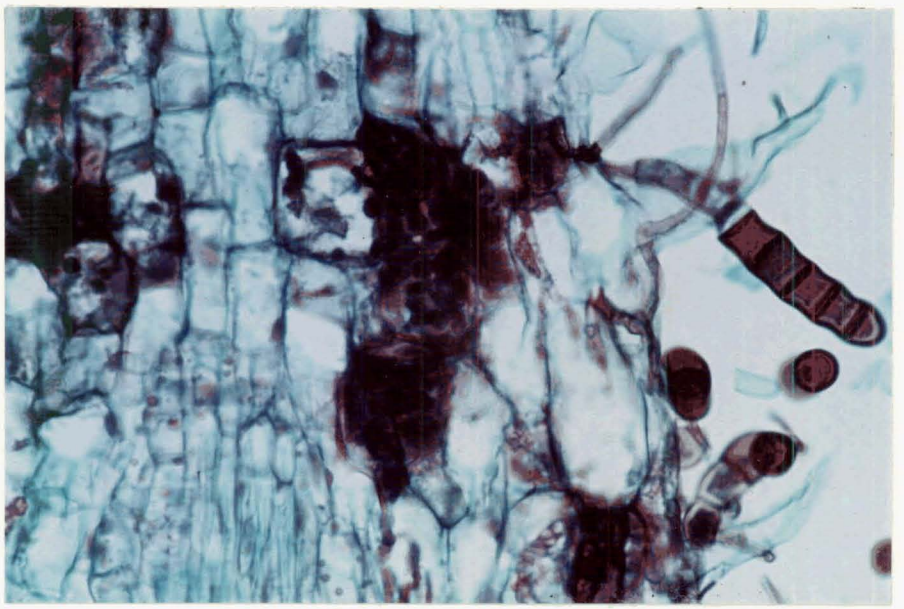
A. Oblique transverse section of root showing groups of cortex cells filled with darkly stained intracellular hyphae of *T. basicola*. Note the production of chlamydospores and endoconidiophores on the root surface. X1,300.

B. Longitudinal section of root showing the darkly stained and partly collapsed walls of infected epidermal and outer cortex cells. Note the presence of lignituber-like outgrowths on the walls of some cells. X1,300.

C. Longitudinal section of root infected on one side with *T. basicola*. Note the absence of *T. basicola* hyphae in cells showing lignituber-like outgrowths on the walls. X1,300.

Plate 4.9

A.



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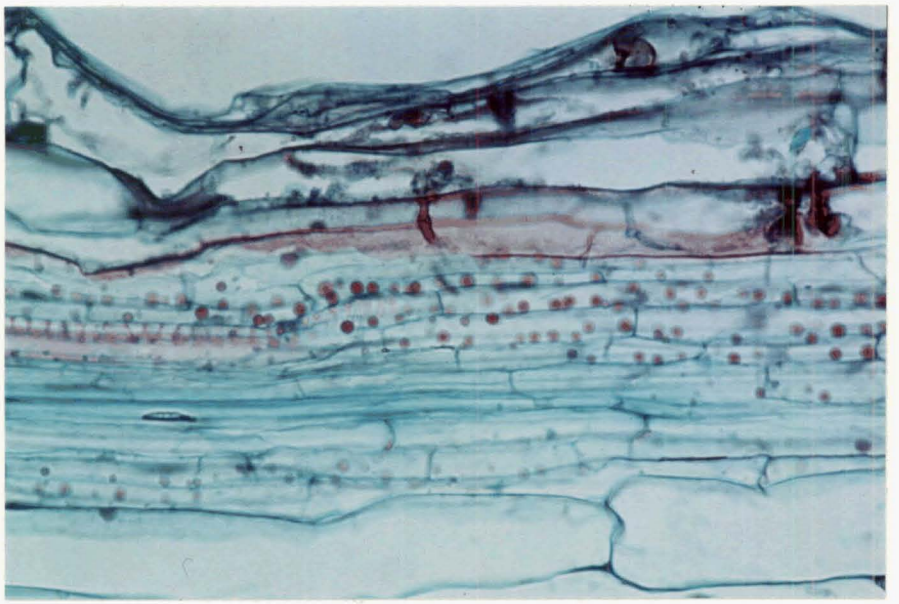
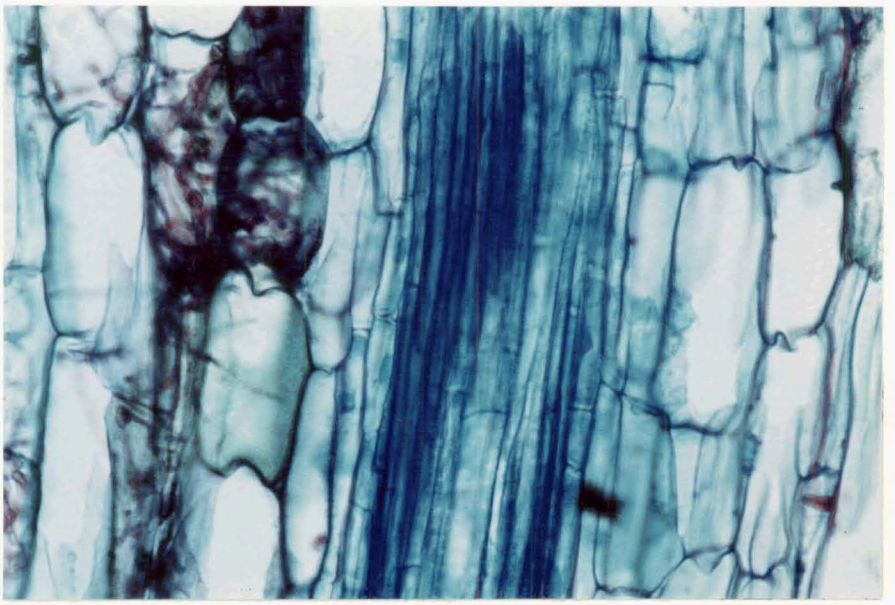


C.



resistant reaction to infection.

F. avenaceum, commonly associated with root rot of subterranean and red clover (Burgess, *et al.*, 1973; Sundheim, 1970), has been shown to cause the death of white clover seedlings under glasshouse conditions by Thornton (1965). Skipp and Christensen (1982) also found that *F. avenaceum* is capable of invading white clover roots grown in the glasshouse although no necrotic lesions are produced on the roots. Under the conditions of this study, hyphae of *F. avenaceum* usually grew extensively in the intercellular spaces and within the cortex and in some instances also in the xylem vessels, resulting in necrotic lesions or discoloration on the roots. Similar extensive development of *F. avenaceum* in the cortex cells and xylem vessels of red clover has been described by Chi *et al.* (1964) and by Siddiqui and Halisky (1968).

F. oxysporum is the most frequently isolated fungus from roots of white clover (Thornton, 1965; Skipp and Christensen, 1982a, b;), red clover (Kilpatrick, 1959; Moody *et al.*, 1967; Sundheim, 1970) as well as subterranean clover (Burgess *et al.*, 1973; McGee & Kellock, 1974). However, not all the tests carried out with *F. oxysporum* under glasshouse conditions had shown it to be pathogenic. Kellock *et al.*, (1978), for instance, found that *F. oxysporum*, although making up 55% of the isolates obtained from subterranean clover seeds, was not pathogenic to subterranean clover seedlings. Similar findings of lack of pathogenicity of *F. oxysporum* on subterranean clover were obtained by Burgess *et al.* (1973) and McGee and Kellock (1974). Thornton (1965), found that only two of the three strains of

F. oxysporum he isolated from white clover roots were pathogenic to white clover seedlings in glasshouse studies. Skipp and Christensen (1982), however, found that both isolates of *F. oxysporum* he used were pathogenic to white clover seedling producing necrotic lesions. In this study, the two isolates were not found to be pathogenic under the conditions of the test.

Thielaviopsis basicola (Berg. & Br.) Ferraris, is well known as the causal agent of serious root rot of tobacco and beans. Another soil borne fungus, *Thielavia basicola*, whose identity had been confused with *Thielaviopsis basicola* in the past, had been shown to attack hundreds of plant species in 18 families (Johnson, 1916), and especially members of the Leguminosae. It is now known that *Thielavia basicola* grows only in association with *Thielaviopsis basicola* which is a separate fungus (Subramanian, 1968). The host plants designated for *Thielavia basicola* are probably host plants of *Thielaviopsis basicola*. It was one of the fungi isolated from white clover roots in the U.S.A. by Kilpatrick (1959). This study shows its ability to invade white clover roots, producing within the root tissues structures similar to those observed in the root tissues of tobacco and bean (Christou, 1962; Pierre & Wilkinson, 1970; Stover, 1950). In all these hosts, three types of hyphae are produced, namely, constricted intracellular hyphae, non-constricted intercellular hyphae and inter- and intracellular reproductive hyphae. The pathogenicity of *T. basicola* on roots of white clover seedlings has also been confirmed in a recent study by Skipp and Christensen (1982b).

CHAPTER 5

INTERACTION STUDIES

5.1 INTRODUCTION

Vesicular-arbuscular mycorrhizal fungi have been shown to be of common occurrence in New Zealand soils (Crush, 1975; Powell, 1977a) and infect most plants growing in them, including white clover (Crush, 1975; Powell & Sithamparanathan, 1977), and are responsible for causing plant growth increases in infertile soils (Powell, 1976). The efficiency of different mycorrhizal fungi in stimulating growth of white clover has been shown to vary in different soils (Powell, 1977b; Powell, 1977c). Previous results have shown that(Chapter 3, experiments 1 & 2) the VA mycorrhiza formed by *G. fasciculatus* causes a growth stimulation of white clover in Tasman and Pawson Hill silt loams over a wide range of phosphorus levels.

Various pathogenic fungi have also been shown to be consistently associated with roots of white clover from different pastures in New Zealand (Menzies, 1973b; Skipp, pers. comm.; Thornton, 1965). The most important of these are *Codinaea fertilis*, *Fusarium* species and *Thielaviopsis basicola*. *C. fertilis* has been shown as the primary pathogen responsible for root rotting and lack of persistence of white clover in the North Island (Menzies, 1973a). *Fusarium* species are the main cause of root rotting and yield reductions in many important legumes overseas (Leath, *et al.*, 1971) and *T. basicola* is the causal agent of root rot of tobacco and bean. In glasshouse

pathogenicity tests carried out in Chapter 4, *C. fertilis*, *F. avenaceum* and *T. basicola* were shown to be capable of invading roots of young white clover seedling grown in Tasman and Pawson Hill silt loams, producing necrotic lesions and causing a reduction in root growth.

Thus far, very little is known about the root rot complex of white clover in New Zealand and the factors involved. According to Menzies (1973b), circumstantial evidence suggests that root rot of white clover could be an unrecognized important problem. As in root rot problems of many other legumes, root rot of white clover is likely to be a product of the interaction of various factors of both the physical and biological environment (Leath *et al.*, 1971).

The role of VA mycorrhizae in plant root diseases has recently received more attention and studies have shown that VA mycorrhizae can cause an increase, decrease or have no effect in disease severity in the different combinations of host-pathogen researched (Schenck & Kellam, 1978). In this study, the role of the VA mycorrhizal fungus *G. fasciculatus* in the root rot syndrome caused by *C. fertilis*, *F. avenaceum* and *T. basicola* was investigated.

Preliminary interaction experiments were carried out using mycorrhizal and non-mycorrhizal plants from the two experiments in Chapter 3. It was found that under the conditions of the experiments, using plants grown in Pawson Hill and Tasman silt loams for 144 and 105 days respectively, little infection was produced on the roots of the plants by *C. fertilis*,

F. avenaceum and *T. basicola*. The conditions of the host plants and the environment play an important part in root rot severity in many legumes. In a grazed pasture, the major factor affecting white clover is the continual defoliation (Menzies, 1973b). Frequent foliage clipping, which simulates grazing, has been shown to cause an increase in root rot severity of white clover by Menzies (1973a), Moody *et al.* (1967) and of red clover by Fezer (1961), Fulton and Hanson (1960) and Siddiqui *et al.* (1968). The interaction of *G. fasciculatus* and the root pathogens *C. fertilis*, *F. avenaceum* and *T. basicola* was first investigated incorporating a foliage clipping treatment to weaken the plants.

The incidence of infection in seedlings inoculated with the three pathogens after germination in the pathogenecity tests (Chapter 4) and the near absence of infection in the much older plants used in the preliminary study suggests that age of seedlings could be an important factor affecting susceptibility of white clover to root rot. According to Yarwood (1959), the susceptibility of plants to disease changes with age. He suggested four categories of relationship between plant age and disease susceptibility:

(1) a transition from susceptibility to resistance with age
(2) increasing susceptibility with age
(3) susceptibility of older and younger plants with most disease resistance at middle life and
(4) resistance in young and old plants with susceptibility in middle age.

Fulton and Hanson (1960) showed that the age of red clover plants affects their susceptibility to root rot caused by various *Fusarium* species, with younger seedlings being more susceptible. An interaction experiment was carried out using mycorrhizal and non-mycorrhizal plants in Tasman silt loam and inoculated with the three pathogens at intervals after transplanting. The root rot severity developed in these plants in the presence and absence of mycorrhiza was assessed. Another experiment was conducted, to determine the effect of using a different inoculum medium and inoculation method in disease development and interaction of the pathogens with mycorrhiza. Plants were grown in Tasman silt loam and the inoculum of each pathogen was introduced before transplanting.

5.2 MATERIALS AND METHODS

5.2.1 General methods

5.2.1.1 Soils

Pawson Hill and Tasman silt loams were used in these studies. The procedure used for the preparation of these soils was as outlined in Chapters 2 and 3.

5.2.1.2 Host plants and inoculation with mycorrhiza and rhizobia

Germination of 'Grasslands Huia' white clover seeds, the transplanting of seedlings, the preparation of mycorrhizal and rhizobia inocula and the inoculation of seedlings was as described in Chapter 2.

5.2.1.3 Pathogen inocula and inoculation methods

Two types of pathogen inocula were used:

(a) Mycelia and spore suspensions

Mycelia and spore suspensions of *C. fertilis*, *F. avenaceum* and *T. basicola* were prepared from mycelial mats of each fungus grown separately in medical flats containing 100 ml of sterilized potato dextrose, potato sucrose and V_8 broth media respectively. Flasks containing *F. avenaceum* were incubated at 20°C in 16 h daylight for 10 days. Those with *C. fertilis* and *T. basicola* were incubated at 25°C in the dark for 14 days. The mycelial mats from each flask were then harvested, washed with distilled water and macerated in distilled water for 1 min in a Waring blender. The suspension was then stab-inoculated with a syringe into the soil around

each seedling, at a rate of 2 ml per stab. Each pot of soil was given 24 ml of the inoculum. Control pots were inoculated with an equal volume of distilled water or autoclaved suspension of each pathogen.

(b) Ground oatmeal and sand cultures

Cultures of *C. fertilis*, *F. avenaceum* and *T. basicola* were grown separately in a mixture of ground oatmeal and sand in Erlenmeyer flasks. The preparation of these cultures and inoculation methods were as described in the pathogenicity tests section in Chapter 4. The soil in each pot was mixed with 50 ml of the inoculum. Control pots were given an equal volume of distilled water or autoclaved ground oatmeal culture of each fungus.

5.2.1.4 Harvesting and assessments

At harvesting, the roots of each plant were carefully washed free of soil with tap water. The root system was then spread out in a white tray with a low level of water. The proportion of roots with disease lesions produced by each pathogen was assessed and expressed as percent root infection. Random samples of roots showing disease symptoms were selected for reisolation studies according to the procedure outlined in the pathogenicity tests section, Chapter 4. The identity of fungi isolated was compared with the original fungi used for inoculation. The shoots and roots were dried in a 105^{o}C oven for 24 h, cooled and weighed separately. In cases where fresh weights of roots were determined, excess water on the roots was carefully removed with tissue paper.

5.2.1.5 Statistical analysis

The results of shoot and root weights and percent root infection were analysed using ANOVA. Transformation of shoot and root weight data into natural logarithm and percent root infection into arcsine was carried out when necessary. Missing plot estimation was conducted where appropriate. In cases where disease lesions are absent among the controls for the pathogens, the inclusion of their percent root infection in the ANOVA would lead to unequal variances for treatments ($var_c=0$). Hence only the pathogen treatments were subjected to ANOVA.

5.2.2 Effect of foliage clipping

The role of the mycorrhizal fungus *G. fasciculatus* on root infection by *C. fertilis*, *F. avenaceum* and *T. basicola* was investigated using plants grown in Pawson Hill silt loam supplied with 9.2 mgP/pot (in 20 ml $Ca(H_2PO_4)_2.H_2O$ solution). A foliage clipping treatment which simulate grazing, was included.

White clover plants were first grown for 70 days with or without mycorrhizal inoculum in 10 cm 'Squat pots' in a 20-25^{o}C growth cabinet with 16 h daylight. Shoots of 35 mycorrhizal and non-mycorrhizal plants were harvested and dry weights determined and compared. The plants were then randomly allocated for inoculation with 24 ml mycelia and spore suspensions of *C. fertilis*, *F. avenaceum*, *T. basicola* or an equal volume of autoclaved suspension of each pathogen and distilled water as controls. The experiment was set up with

a 7 x 2 factorial design, and laid out in five complete randomised blocks. The treatments were:

Pathogen: Three pathogens with respective autoclaved mycelia and spore suspension as controls and one distilled water control

Mycorrhizal: Inoculated or uninoculated

Replicates: 5

After inoculation, the pots were returned to the growth cabinet. Shoots were clipped four times within a 25 days period, beginning at 14 days after inoculation. The experiment was terminated 35 days after the final clipping. Disease symptoms on the roots were noted. Shoot and root dry weights and percent root infection caused by the pathogens were determined. For a comparison of the effect of each pathogen on mycorrhizal and non-mycorrhizal plants, the ratios of shoot or root weight of pathogen inoculated and control plants were estimated and their standard errors calculated as in Chapter 3. These ratios were then compared using linear comparison of means as described previously.

5.2.3 Effect of plant age

The influence of VA mycorrhiza on root rot development of white clover seedlings, inoculated at various time intervals with the three pathogens, was investigated.

Seedlings were first grown in Tasman silt loam (applied with 92.0 mgP/pot in 20 ml $Ca(H_2PO_4)_2.H_2O$ solution) with or without *G. fasciculatus* in 10 cm 'Squat pots' for 28, 42 and 56 days in a 20-25^{o}C growth cabinet with 16 h daylight. The shoots of 20 mycorrhizal and non-mycorrhizal plants of each age group were harvested, dried and weighed as described previously.

Seedlings were germinated for 48 h and transplanted into pots of soil with or without *G. fasciculatus* by the method described previously. These and the other seedlings were inoculated with 24 ml of a macerated mycelia and spore suspension of each of the three pathogens. Control pots were given an equal volume of distilled water. The experiment was set up with a 4 x 4 x 2 factorial design, laid out in five complete randomised blocks. The treatments were:

Plant age:	0, 28, 42 and 56 days after transplanting
Pathogen:	Three root pathogens and one distilled water control
Mycorrhizal:	Inoculated or uninoculated
Replicates:	5

The pots were returned to the growth cabinet and the experiment terminated after 35 days. The plants were harvested and roots observed for disease symptoms. Shoot dry weight, root fresh weight and percent root infection was determined. Random samples of roots showing disease symptoms were fixed in 3% glutaraldehyde made up in 0.1 M sodium phosphate buffer at pH 6.8, dehydrated and embedded according to the procedure outlined in Appendix 2. The samples were then sectioned (10-15 μm thick), mounted onto slides, stained with safranin-fast green (Appendix 3) or periodic acid-Schiff's stain (Appendix 4) and examined under the optical microscope.

5.2.4 Effect of changing pathogen inoculum media

In the interaction studies conducted thus far, macerated mycelia and spore suspensions of the pathogens were used. These were introduced by stab-inoculation into soil surrounding the roots at various locations. In some cases, the amount of infection produced in the roots varied greatly for plants in the same treatments. Grogan *et al.* (1980) suggested that for fungal propagules that are randomly distributed, some would touch the rhizoplane and others would be located in the soil surrounding the root surface, but only those within a limited distance from the root surface would be able to cause infection. In this study, an inoculation method was employed, to ensure a more even distribution of pathogen inocula throughout the soil system.

The inoculum of *C. fertilis*, *F. avenaceum* and *T. basicola* consisted of cultures grown separately in a mixture of ground oatmeal and sand medium. The preparation of the inoculum and inoculation method is described in the general methods section. Tasman silt loam (applied with 214.7 mgP/pot in 20 ml $Ca(H_2PO_4)_2.H_2O$ solution) was used and potted in 10 cm 'Squat pots'. Seedlings were transplanted 48 h after germination. A 7 x 2 factorial design was used and laid out in five complete randomised blocks. The treatments were:

Pathogen: Three pathogens with respective autoclaved cultures as controls and one distilled water control

Mycorrhizal: Inoculated or uninoculated

Replicates: 5

The experiment was housed in a glasshouse with air temperature fluctuating between 10-30^{o}C, and terminated after 42 days. The plants were harvested, roots washed free of soil and assessed for percent root infection. Shoot dry weight and root fresh weight were determined as described previously. Random samples of roots were processed for reisolation and microscopic studies as described previously. The remaining root systems in the mycorrhizal treatment were cleared and stained with lactophenol trypan blue as outlined in Appendix 1. Mycorrhizal root infection was estimated by spreading roots out in a 15 cm petri-dish marked with gridlines, examining under a stereomicroscope and noting the presence or absence of infection at each of a 100 root/gridline intersect points for each plant.

5.3 RESULTS

5.3.1 Effect of foliage clipping

White clover plants inoculated with *G. fasciculatus* and grown for 70 days in Pawson Hill silt loam had significantly greater shoot dry weight than the uninoculated plants ($P<0.001$) (Table 5.1).

Table 5.1 Shoot dry wt. of white clover grown for 70 days in the presence (M) or absence (N) of *G. fasciculatus* in Pawson Hill silt loam.

Shoot dry wt. (mg)	
M	N
5.384 (216.8)	4.063 (57.2)

S.E.M. = 0.096

Data $\log_e$ transformed

Backtransformed data in parentheses

Roots of mycorrhizal and non-mycorrhizal plants inoculated with *C. fertilis*, *F. avenaceum* and *T. basicola* and harvested 35 days after the final clipping treatment showed root disease symptoms typical of infection by the three pathogens. Dark brown discoloration or lesions appeared in the top and lateral roots and in some cases severe rotting and reduction of root systems occurred. The three pathogens were also consistently isolated from infected roots showing disease symptoms. Control plants not inoculated with the pathogens had a small proportion of their root tips turned brown as a result of the clipping treatment but none of them showed typical root disease symptoms of the pathogens, nor were any

of the pathogens isolated from them.

The percent root infection produced by the pathogens on inoculated plants is presented in Table 5.2. The percent root infection produced by *T. basicola* on non-mycorrhizal plants was significantly greater than that produced on the roots of mycorrhizal plants. Differences in percent root infection produced by *C. fertilis* and *F. avenaceum* on mycorrhizal and non-mycorrhizal plants are not significant.

Table 5.2 Percent root infection produced by *C. fertilis* (CF), *F. avenaceum* (FA) and *T. basicola* (TB) on mycorrhizal (M) and non-mycorrhizal (N) plants in Pawson Hill silt loam.

Fungal treatment	Percent root infection (%) M	N
CF	14.6	17.8
FA	8.6	6.8
TB	1.4	30.8

S.E.M. = 6.69

L.S.D. (5%) = 19.1

Table 5.3 shows the shoot and root yields and relative reduction in yields at the final harvest of mycorrhizal and non-mycorrhizal plants treated with the pathogens and controls. Differences in the results of controls using distilled water or autoclaved pathogen inocula are not significant. Hence only those of the latter are presented for comparison.

Table 5.3 Shoot and root dry wt. and relative reductions in wt. of mycorrhizal (M) and non-mycorrhizal (N) plants inoculated with *C. fertilis* (CF), *F. avenaceum* (FA), *T. basicola* (TB) and controls (CFC, FAC, TBC).

	Shoot dry wt. (mg)		*Rel. reduction in shoot dry wt. (%)		Root dry wt. (mg)		*Rel. reduction in root dry wt. (%)	
Fungal treatment	M	N	M	N	M	N	M	N
CF	564.0	89.0	39	88	173.0	62.6	47	64
CFC	923.4	721.0			328.4	175.0		
FA	704.6	411.2	6	35	251.2	131.2	8	34
FAC	750.4	634.2			273.0	197.4		
TB	671.2	295.4	23	53	318.0	102.6	-	29
TBC	874.8	624.2			279.4	145.0		
	S.E.M. = 131.0				S.E.M. = 46.84			
	L.S.D. (5%) = 373.2				L.S.D. (5%) = 132.5			

*Relative reduction in shoot or root dry wt. = $\frac{C-S}{C}$ x 100%

where C = shoot or root dry wt. of controls

S = shoot or root dry wt. of plants inoculated with the pathogens.

There were no significant differences between the shoot dry weights of mycorrhizal and non-mycorrhizal plants inoculated with *F. avenaceum* or the controls for each of the three pathogens (Table 5.3). Non-mycorrhizal plants inoculated

with *C. fertilis* and *T. basicola*, however, had significantly smaller shoot dry weights than the mycorrhizal plants in the same treatments. The root dry weights of mycorrhizal plants inoculated with *C. fertilis*, *F. avenaceum* and its control were not significantly different from the corresponding weight of non-mycorrhizal plants. Root dry weight of mycorrhizal plants treated with *T. basicola* and controls for *C. fertilis* and *T. basicola* were significantly greater than those of non-mycorrhizal plants.

The relative reductions in shoot and root dry weight of the non-mycorrhizal plants treated with each pathogen were apparently larger than the corresponding values for the mycorrhizal plants (Table 5.3). To enable a comparison of the effects of pathogens on mycorrhizal and non-mycorrhizal plant yield, the ratios of shoot or root yield of pathogen treated and control plants were also estimated and shown in Table 5.4.

Table 5.4 Relative shoot and root dry wt. of mycorrhizal (M) and non-mycorrhizal (N) plants treated with *C. fertilis* (CF), *F. avenaceum* (FA), *T. basicola* (TB) and the respective controls.

Fungal treatment	*Realtive shoot dry wt.		*Relative root dry wt.	
	M	N	M	N
CF	0.611(0.1453)	0.123(0.0618)	0.527(0.1097)	0.358(0.1818)
FA	0.940(0.1856)	0.648(0.3062)	0.920(0.2462)	0.665(0.3009)
TB	0.767(0.1842)	0.473(0.2396)	1.138(0.2927)	0.708(0.2982)

S.E.M. in parentheses.

* Relative shoot or root dry wt. = $\frac{s}{c}$

where s = shoot or root dry wt. of plants inoculated with the pathogens

c = shoot or root dry wt. of plants inoculated with autoclaved pathogen inocula.

There is a significant overall difference between the relative shoot weights of mycorrhizal and non-mycorrhizal plants ($P<0.05$), with the values being greater for mycorrhizal plants in each treatment. When the results for each pathogen were analysed separately, only the difference for *C. fertilis* was significant ($P<0.01$) but not the differences for *F. avenaceum* and *T. basicola* (Table 5.4).

The relative root dry weights of mycorrhizal plants also appeared to be greater than the non-mycorrhizal plants for each fungal treatment (Table 5.4). These differences, however, are not significant when the results were analysed separately or together by using linear comparison of means.

5.3.2 Effect of plant age

Shoot dry weight of mycorrhizal plants are significant ly greater ($P<0.001$) than that of non-mycorrhizal plants at 28, 42 and 56 days after transplanting (Table 5.5).

Table 5.5 Shoot dry wt of mycorrhizal (M) and non-mycorrhizal (N) plants at 28, 42 and 56 days after transplanting.

Days after transplanting	* Shoot dry wt. (mg) M	N
28	2.718 (14.1)	1.524 (3.6)
	S.E.M. = 0.090	
42	4.490 (88.1)	1.609 (4.0)
	S.E.M. = 0.097	
56	6.321 (555.1)	2.627 (12.8)
	S.E.M. = 0.139	

* Shoot dry wt. data $\log_e$ transformed.

Backtransformed data in parentheses.

The magnitude of mycorrhizal response is shown in Table 5.6. There is a significant linear trend ($P<0.01$) in the increase in magnitude of mycorrhizal response from 28 to 56 days. Comparisons made between individual pairs of results indicate that the increase from 28 to 42 days is significant ($P<0.001$), while that between 42 and 56 days is not significant.

Table 5.6 Magnitude of mycorrhizal response (M/N) for plants at 28, 42 and 56 days after transplanting into Tasman silt loam.

Days after transplanting	* Magnitude of mycorrhizal response M/N
28	3.8 (0.576)
42	20.5 (3.099)
56	32.5 (9.598)

S.E.M. in parentheses

* Magnitude of mycorrhizal response = M/N

where M = shoot dry wt. of mycorrhizal plants

N = shoot dry wt. of non-mycorrhizal plants.

Roots of mycorrhizal and non-mycorrhizal white clover inoculated with the three pathogens at various intervals show varying degrees of root rotting and necrosis, with seedlings inoculated at day 0 showing the most severe rot symptoms. The diseased roots yielded the original fungi used for inoculation, when plated onto acidified agar plates.

Diseased roots of mycorrhizal and non-mycorrizal plants were also observed under the microscope to contain mycelia of each pathogen. In the case of *C. fertilis*, there was massive development of hyphae in some regions of the roots. Lignituber-like outgrowths, however, were commonly observed on the walls of epidermal and outer cortical cells, adjacent to cells containing *G. fasciculatus* (Plate 5.1A). There was little penetration and colonization of the inner cell layers in these cases. For *F. avenaceum*, there was massive development of hyphae in some isolated groups of cells in the lateral (Plate 5.1B) and tap roots of mycorrhizal plants. Hyphae and chlamydospores of *T. basicola* were observed in cells adjacent to those containing arbuscules or vesicles of *G. fasciculatus* in the roots (Plate 5.2A). In severe cases of infection, hyphae and chlamydospores of *T. basicola* were also observed in the xylem vessels or parenchyma cells of the vascular tissues of mycorrhizal roots (Plate 5.2B).

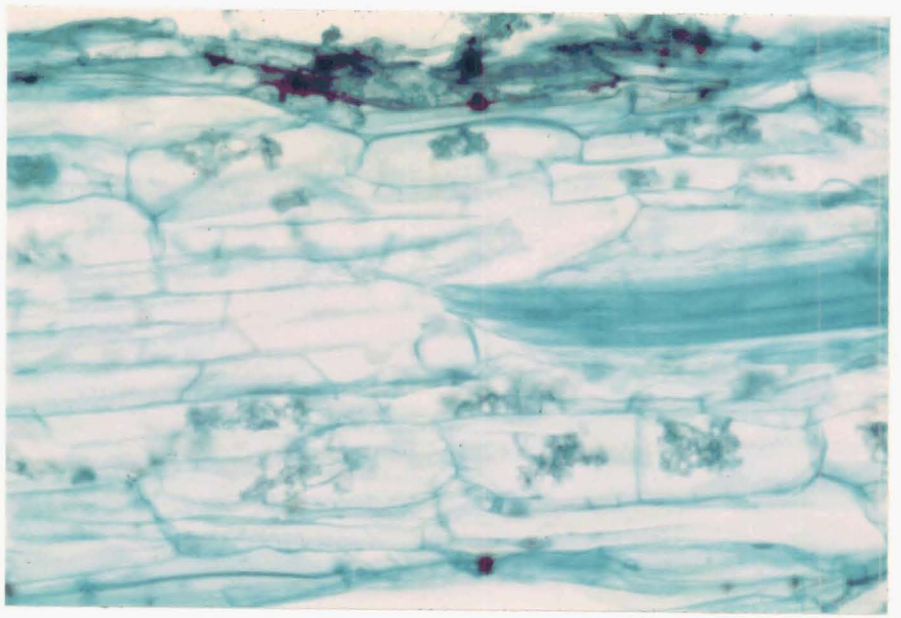
Plate 5.1 Infection and colonization of roots of mycorrhizal plants by *C. fertilis* and *F. avenaceum*. Roots cut longitudinally.

A. Section of mycorrhizal root showing lignituber-like outgrowths on the walls of cells in response to *C. fertilis* penetration. Note the presence of arbuscules in the inner cortical cells. Section stained with safranin-fast green. X1,300.

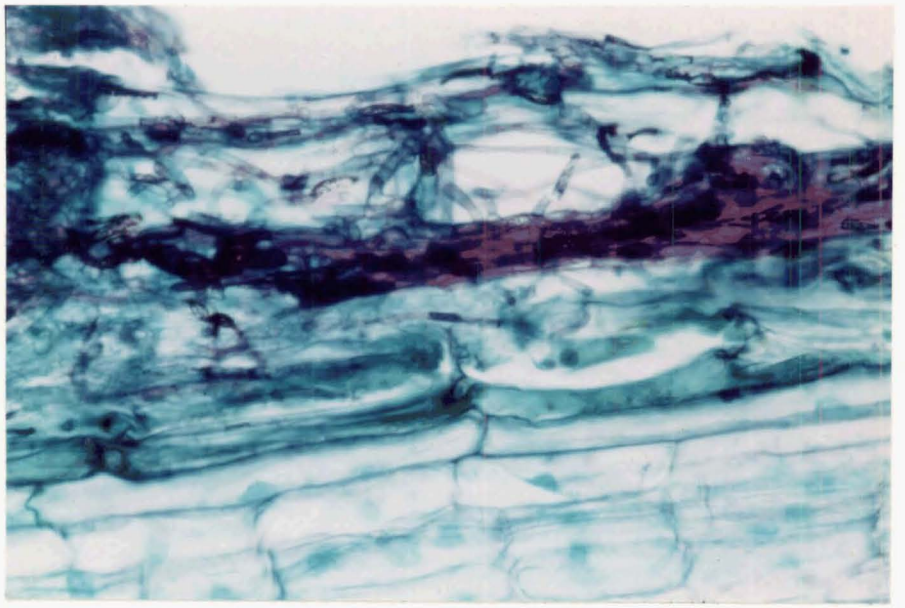
B. Section showing massive development of *F. avenaceum* hyphae in the epidermal and outer cortical cells of a lateral root of mycorrhizal plant. Structures of *G. fasciculatus* were not observed in this root segment. Section stained with periodic acid-Schiff's stain. X1,300.

C. Section through the cortical cells of a tap root showing massive development of hyphae of *F. avenaceum* within the cells. Section stained with periodic acid-Schiff's stain. X1,300.

A.



B.



C.

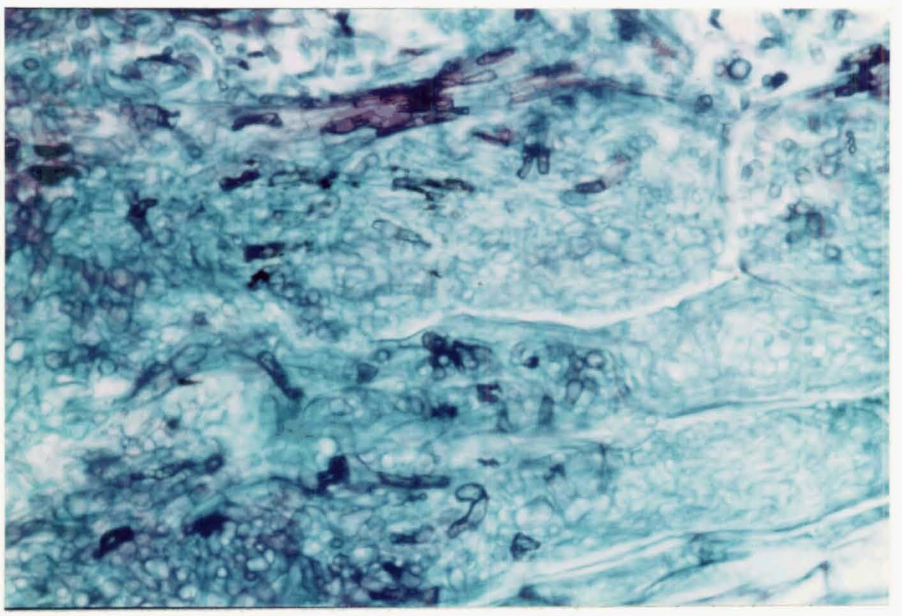


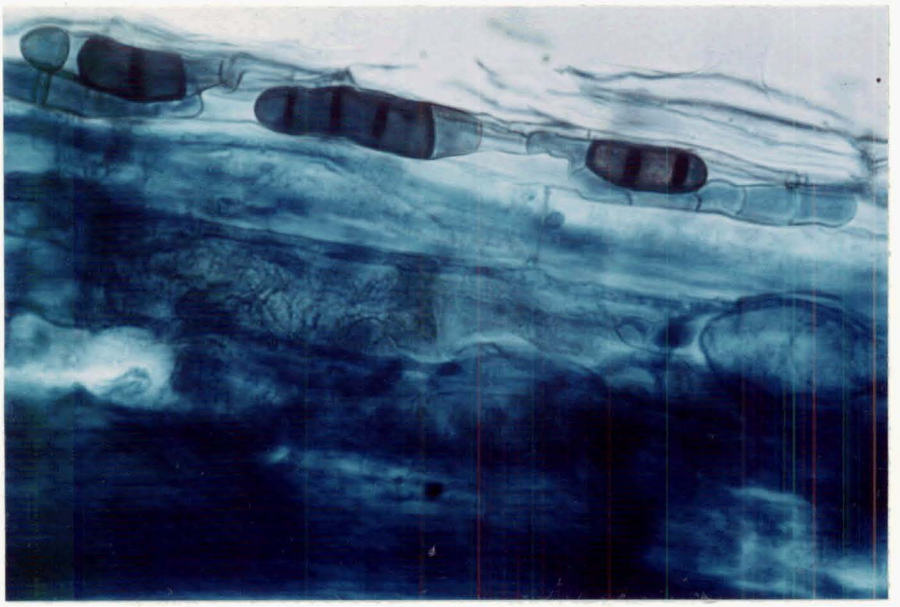
Plate 5.2 Infection and colonization of roots of mycorrhizal plants by *T. basicola*.

A. Whole mount of mycorrhizal root showing chlamydospores and hyphae in the cortical cells. Note the presence of a vesicle in an adjacent cell. Root cleared and stained with lactophenol trypan blue. X1,300.

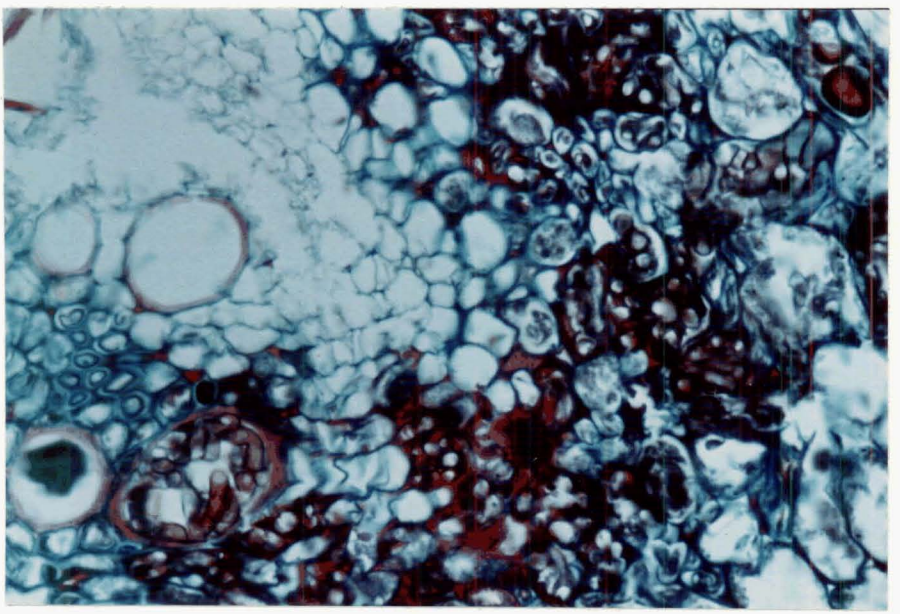
B. Cross section of a mycorrhizal root showing the colonization of xylem vessels and parenchyma cells in the vascular tissues by hyphae and chlamydospores of *T. basicola*.
Section stained with safranin-fast green.
X1,300.

Plate 5.2

A.



B.



The results of shoot and root yields and percent root infection of the pathogens for each plant age group were analysed separately because of heterogeneous error variances when analysed together by ANOVA.

Table 5.7 shows the shoot and root yields, relative reductions in yields and percent root infection of each pathogen on plants inoculated at transplanting (day 0). Shoot and root yields of mycorrhizal plants are significantly greater than the non-mycorrhizal plants inoculated with the respective pathogens, except for root weight differences of *T. basicola* treatment (Table 5.7).

Shoot dry weights of mycorrhizal and non-mycorrhizal were reduced by infection with each of the pathogens. Large relative reductions in shoot dry weight were obtained for plants inoculated with each pathogen, in particular, for the non-mycorrhizal plants.

Root fresh weights of mycorrhizal plants are significantly reduced by infection with *T. basicola* (5% level) and *F. avenaceum* (10% level) but not by *C. fertilis*. Root fresh weight of non-mycorrhizal plants are significantly reduced by *C. fertilis* (5% level), *F. avenaceum* and *T. basicola* (both at 10% level). Differences in percent root infection produced on mycorrhizal and non-mycorrhizal plants by *F. avenaceum* and *T. basicola* are not significant. *C. fertilis*, however, produced significantly greater percent root infection on non-mycorrhizal plants compared to mycorrhizal plants (Table 5.7).

Table 5.7 Shoot dry wt., root fresh st., relative reduction in shoot dry wt. and root fresh wt. and percent root infection of mycorrhizal (M) and non-mycorrhizal (N) plants inoculated with *C. fertilis* (CF), *F. avenaceum* (FA), *T. basicola* (TB) and control (C) at transplanting (day 0).

Fungal Treatment	Shoot dry wt.(mg)		Relative reduction in shoot dry wt.(%)		*Root fresh wt.(g)		Relative reduction in root fresh wt.(%)		Percent root infection (%)	
	M	N	M	N	M	N	M	N	M	N
CF	39.6	4.8	26.9	62.5	4.85(126.7)	2.57(12.0)	48.2	78.2	12.2	66.0
FA	38.7	6.5	28.6	49.2	4.29 (72.0)	2.86(16.5)	70.6	70.0	18.0	13.3
TB	30.2	6.7	44.3	47.7	3.78 (42.9)	2.69(13.7)	82.5	75.1	55.6	37.1
C	54.2	12.8			5.50(244.8)	4.02(55.0)				

S.E.M. = 7.03
L.S.D.(5%) = 20.3

S.E.M. = 0.47
L.S.D.(5%) = 1.36
L.S.D.(10%) = 1.13

S.E.M. = 12.1
L.S.D.(5%) = 36.0

* Root fresh wt. data $\log_e$ transformed.

Backtransformed data in parentheses.

Root disease symptoms were present on the roots of mycorrhizal and non-mycorrhizal plants inoculated at day 28 with the pathogens. Root rotting, however, appeared to be less severe in most treatments, compared to plants inoculated at day 0. Table 5.8 shows the shoot and root weight and relative reduction in weight of these plants at harvesting. Shoot and root weights of mycorrhizal plants are significantly greater than those of non-mycorrhizal plants in the respective pathogen and control treatments. There were no significant differences in the shoot and root yields of mycorrhizal or non-mycorrhizal plants treated with the pathogens compared to the control, except for shoot yields of non-mycorrhizal plants treated with *F. avenaceum*. Relative reduction in shoot and root weight caused by each of the pathogens appeared to be larger for the non-mycorrhizal plants (Table 5.8).

C. fertilis and *F. avenaceum* produced significantly greater percent root infection on non-mycorrhizal than on mycorrhizal plants. Percent root infection produced by *T. basicola* on mycorrhizal and non-mycorrhizal plants are not significantly different (Table 5.8).

Table 5.9 shows the shoot and root weights, relative reduction in weights and percent root infection of plants inoculated with the pathogens 42 days after transplanting. Shoot and root weights of mycorrhizal plants are significantly greater than those of non-mycorrhizal plants. The pathogens, however, appeared to have very little effect on the yields of both the mycorrhizal and non-mycorrhizal plants. Shoot and root weights of non-mycorrhizal plants are significantly

Table 5.8 Shoot dry wt., root fresh wt., relative reduction in shoot dry wt. and root fresh wt. and percent root infection of mycorrhizal (M) and non-mycorrhizal (N) plants inoculated with *C. fertilis* (CF), *F. avenaceum* (FA), *T. basicola* (TB) and control (C) 28 days after transplanting.

	*Shoot dry wt.(mg)		Relative reduction in shoot dry wt.(%)		Root fresh wt.(mg)		Relative reduction in root fresh wt.(%)		Percent root infection (%)	
Treatment	M	N	M	N	M	N	M	N	M	N
CF	5.25(189.9)	2.49(11.1)	23.4	38.3	744.4	40.6	7.1	54.5	5.8	16.2
FA	5.37(214.6)	1.99 (6.3)	13.4	65.0	686.3	26.8	14.4	70.0	2.4	24.0
TB	5.40(219.5)	2.49(11.1)	11.4	38.3	830.5	75.5	-	15.4	15.6	7.6
C	5.52(247.8)	2.95(18.0)			801.7	89.2				

S.E.M. = 0.229
L.S.D. (5%) = 0.66

S.E.M. = 70.30
L.S.D. (5%) = 203.6

S.E.M. = 3.10
L.S.D.(5%) = 9.0

*Shoot dry wt. data $\log_e$ transformed.
Backtransformed data in parentheses.

reduced only by *F. avenaceum* (Table 5.9). Percent root infection produced on non-mycorrhizal plants by *C. fertilis* and *F. avenaceum* are significantly greater than that on mycorrhizal plants, while that produced by *T. basicola* are not significantly different.

Results of the effect of pathogens on plant yields and percent root infection of plants inoculated 56 days after transplanting are shown in Table 5.10. Shoot and root yields of mycorrhizal plants are significantly greater than those of non-mycorrhizal plants in the respective pathogen treatment. The reductions in shoot and root yields of mycorrhizal plants infected with *C. fertilis* and *F. avenaceum* are significant (at 5% level). The reduction in shoot yield of mycorrhizal plants by *T. basicola* is significant (at 10% level) but not the root yield. Differences in the shoot and root yields of non-mycorrhizal plants treated with each pathogen and the control are not significant. No significant differences were found in the percent root infection produced on mycorrhizal and non-mycorrhizal plants by *C. fertilis* or *T. basicola*. *F. avenaceum*, however, produced a significantly higher percent root infection on non-mycorrhizal plants (Table 5.10).

5.3.3 Effect of changing pathogen inoculum media

Root disease symptoms were observed on the roots of mycorrhizal and non-mycorrhizal plants inoculated with each of the three pathogens. For plants inoculated with *C. fertilis*, infected roots showed mainly light brown surface discoloration, with tiny necrotic spots or dark brown lesions of limited sizes scattered over the roots. Under the stereo-

Table 5.9 Shoot dry wt., root fresh wt., relative reduction in shoot dry wt. and root fresh wt. and percent root infection of mycorrhizal (M) and non-mycorrhizal (N) plants inoculated with *C. fertilis* (CF), *F. avenaceum* (FA), *T. basicola* (TB) and control (C) 42 days after transplanting.

	* Shoot dry wt.(mg)		Relative reduction in shoot dry wt.(%)		* Root fresh wt.(mg)		Relative reduction in root fresh wt(%)		Percent root infection (%)	
Treatment	M	N	M	N	M	N	M	N	M	N
CF	6.35(570.1)	2.87(16.7)	3.5	-	7.09(1197.5)	4.03 (55.3)	23.2	18.2	4.4	10.4
FA	6.34(564.4)	2.19 (7.9)	4.4	42.3	7.10(1211.6)	3.32(26.7)	22.3	60.5	2.2	12.6
TB	6.42(615.8)	3.51(32.3)	-	-	7.18(1310.3)	4.93(137.4)	16.0	-	7.4	3.0
C	6.38(590.5)	2.69(13.7)			7.35(1559.6)	4.23 (67.6)				
	S.E.M. = 0.16				S.E.M. = 0.18				S.E.M. = 1.71	
	L.S.D.(5%) = 0.48				L.S.D.(5%) = 0.52				L.S.D.(5%) = 5.0	

* Data $\log_e$ transformed.

Backtransformed data in parentheses.

Table 5.10 Shoot dry wt., root fresh wt., relative reduction in shoot dry wt. and root fresh wt. and percent root infection of mycorrhizal (M) and non-mycorrhizal (N) plants inoculated with *C. fertilis* (CF), *F. avenaceum* (FA), *T. basicola* (TB) and Control (C) 56 days after transplanting.

	Shoot dry wt.(mg)		Relative reduction in shoot dry wt.(%)		Root fresh wt.(mg)		Relative reduction in root fresh wt.(%)		* Percent root infection (%)	
Treatment	M	N	M	N	M	N	M	N	M	N
CF	838.8	37.7	19.4	32.1	1557.1	122.9	33.3	52.5	0.48(21.3)	0.32(10.1)
FA	736.6	94.5	29.2	-	1522.6	194.5	34.7	24.8	0.12 (1.5)	0.39(14.6)
TB	877.6	101.7	15.6	-	1938.7	327.5	16.9	-	0.22 (4.9)	0.16 (2.6)
C	1040.2	55.5			2333.3	258.8				

S.E.M. = 60.0
L.S.D.(5%) = 173.6
L.S.D.(10%) = 144.2

S.E.M. = 169.9
L.S.D.(5%) = 490.5

S.E.M. = 0.09
L.S.D.(5%) = 0.27

* Percent root infection data arcsine transformed.

Backtransformed data (X100) in parentheses.

microscope, necrotic lesions were observed to consist of dead collapsed epidermal and outer cortical cells. Thin sections of discolored roots showed the common occurrence of lignituber-like outgrowths on the walls of many epidermal and outer cortical cells. Penetration and colonization of these cells by *C. fertilis* appeared to be limited. Similar lignituber-like outgrowths were present on the walls of cells in mycorrhizal roots.

Symptoms of infection produced by *F. avenaceum* on the roots of mycorrhizal and non-mycorrhizal plants are very similar (Plate 5.3A). Hyphae of *F. avenaceum* were observed in isolated groups of outer cortical cells in both the mycorrhizal and non-mycorrhizal plants. In most cases, there was little penetration into the inner cell layers.

Mycorrhizal and non-mycorrhizal roots infected with *T. basicola* showed light brown discoloration with darker brown necrotic lesions scattered over the root systems (Plate 5.3B). Under the microscope, the lesions were observed to consist of dead collapsed epidermal cells, while sparse hyphae of *T. basicola* occurred in the cells of discolored roots of mycorrhizal plants. With the non-mycorrhizal roots, there appeared to be a more profuse development of hyphae within the cortical cells.

Table 5.11 shows the results of the effect of *C. fertilis*, *F. avenaceum* and *T. basicola* on plant yields and root infection. No significant differences were found between the percent root infection of each pathogen on mycorrhizal and non-mycorrhizal plants. With the exception of *C. fertilis*,

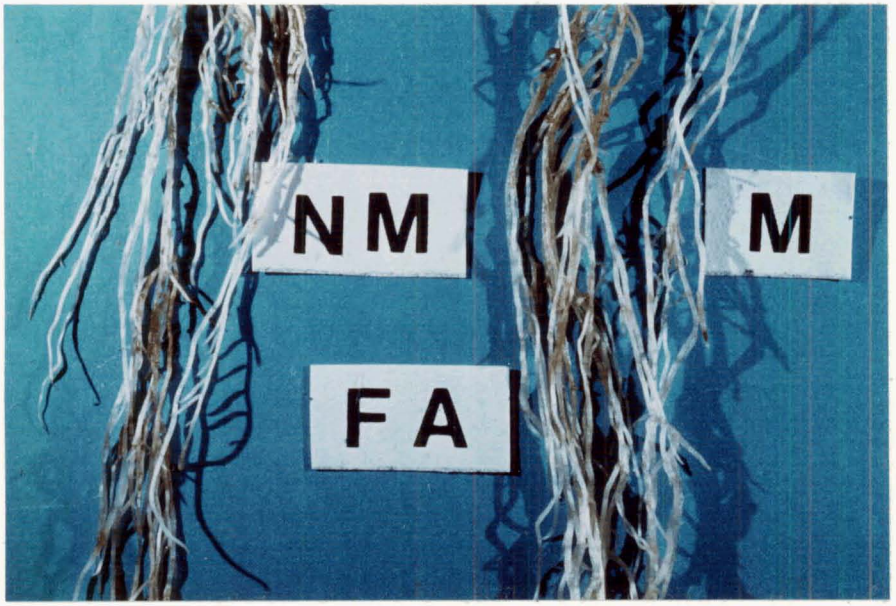
Plate 5.3 Symptoms of root infection produced on the roots of mycorrhizal and non-mycorrhizal by *F. avenaceum* and *T. basicola*.

A. Necrotic lesions and discoloration produced on the roots of mycorrhizal and non-mycorrhizal plants inoculated with *F. avenaceum* at transplanting. X3.5.

B. Brown discoloration and lesions produced on the roots of mycorrhizal and non-mycorrhizal plants inoculated with *T. basicola* at transplanting. X3.5.

Plate 5.3

A.



B.

the shoot and root weights of mycorrhizal plants were significantly greater than those of non-mycorrhizal plants in the same pathogen treatment. A significant reduction in shoot dry weight of non-mycorrhizal plants was caused by *T. basicola* when compared to the distilled water controls. Significant reduction in root fresh weight of mycorrhizal plants was produced by *C. fertilis* and in non-mycorrhizal plants by *T. basicola* (Table 5.11). With the exception of the significant reduction in root fresh weight of mycorrhizal plants by *C. fertilis*, the pathogens appeared to have no effect on shoot or root yields of mycorrhizal plants.

Mycorrhizal root infection of plants treated with *C. fertilis* was significantly less than that of the distilled water controls but not different from the autoclaved inoculum treatment. Mycorrhizal root infection in plants inoculated with *F. avenaceum* and *T. basicola* were not significantly different from the distilled water control but significantly greater than the respective autoclaved inocula treatment.

Table 5.11 Shoot dry wt., root fresh wt., relative reduction in shoow dry wt. and root fresh wt., mycorrhizal root infection and percent root infection of mycorrhizal (M) and non-mycorrhizal (N) plants inoculated with *C. fertilis* (CF), *F. avenaceum* (FA), *T. basicola* (TB) and controls (CFC, FAC, TBC, C).

	**Shoot dry wt.(mg)		*Relative reduction in shoot dry wt.(%)		**Root fresh wt.(mg)		*Relative reduction in shoot fresh wt.(%)		Percent root infection (%)		Mycorrhizal root infection (%)
Treatment	M	N	M	N	M	N	M	N	M	N	
CF	3.87(46.9)	3.44(30.2)	5	-	5.09(161.4)	4.75(114.6)	38	22	16.7	10.0	14.7
CFC	3.92(49.4)	3.35(27.5)			5.56(258.8)	5.00(147.4)					21.0
FA	4.04(55.8)	2.72(14.2)	1.1	28	5.72(303.9)	4.35(76.5)	-	-	19.2	10.8	34.7
FAC	4.05(56.4)	3.03(19.7)			5.69(294.9)	4.34(75.7)					17.3
TB	4.31(73.4)	2.37(9.7)	-	48	5.76(316.3)	3.90(48.4)	-	51	17.5	10.8	48.8
TBC	3.88(47.4)	3.00(19.1)			5.55(256.2)	4.60(98.5)					22.7
C	4.16(63.1)	2.89(17.0)			5.87(354.2)	4.76(115.7)					34.5
	S.E.M. = 0.20				S.E.M. = 0.24				S.E.M. = 4.4		S.E.M. = 3.96
	L.S.D.(5%) = 0.57				L.S.D.(5%) = 0.68				L.S.D.(5%) = 13.0		L.S.D.(5%) = 11.4

* Relative reduction in shoot dry wt. or root fresh wt. = $\frac{C_s - S}{C_s} \times 100\%$

where C_s = shoot dry wt. or root fresh wt. of plants inoculated with autoclaved pathogen inoculum.

S = shoot dry wt. or root fresh wt. of plants inoculated with pathogen.

** Shoot dry wt. and root fresh wt. data $\log_e$ transformed.

Backtransformed data in parentheses.

5.4 DISCUSSION

The results of these interaction studies show that roots of white clover were invaded by each of the three fungi *C. fertilis*, *F. avenaceum* and *T. basicola*. The extent of penetration and colonization of the host roots, and the detrimental effect of the pathogens varied according to the experimental conditions.

With frequent clipping *C. fertilis* and *F. avenaceum* caused extensive root rotting and necrosis, in both mycorrhizal and non-mycorrhizal plants inoculated 70 days after transplanting. There was also considerable reduction in shoot and root yields. With *T. basicola*, there was very little damage on the roots of mycorrhizal plants. Roots of non-mycorrhizal plants were, however, severely affected.

The presence of necrotic lesions and reduction in shoot and root yields indicates that the mycorrhizal fungus does not confer resistance to root infection by the three pathogens under the described conditions. For *C. fertilis*, root dry weights and percent root infection of mycorrhizal and non-mycorrhizal plants are not significantly different (Tables 5.2, 5.3). Mycorrhizal plants, however, continue to give a significantly greater shoot yield. This was found to correspond to a greater relative reduction in shoot dry weight of 88% in non-mycorrhizal plants, compared to 39% in the mycorrhizal plants. Hence, it appears that white clover in the presence of VA mycorrhiza infection is more tolerant to the detrimental effect of *C. fertilis* in respect to shoot yield.

With *F. avenaceum*, there were no significant differences between the mycorrhizal and non-mycorrhizal plants in the percent root infection. Non-mycorrhizal plants, however, appear to be more severely affected in their shoot and root yields. A relative reduction in shoot and root dry weight of 35% and 34% respectively was obtained for the non-mycorrhizal plants compared to 6% and 8% for the mycorrhizal plants. This suggests again that the VA mycorrhiza reduces the damaging effect of *F. avenaceum* on white clover plant yield.

A similar beneficial effect of the presence of the mycorrhizal fungus could be extended to infection by *T. basicola*. Mycorrhizal plants had significantly greater shoot and root dry weights than the non-mycorrhizal plants, and also a smaller relative reduction in shoot and root weight. The lack of statistical significance in large differences between the relative shoot and root yields (Table 5.5) of mycorrhizal and non-mycorrhizal plants in the *T. basicola* and *F. avenaceum* treatments is probably due to the variability in the experimental system. One contributing factor may be the variation in the amount of infection produced by the pathogens in different plants of the same treatment. An example is non-mycorrhizal plants inoculated with *T. basicola*, where percent root infection ranges from 2 to 77%.

Mycorrhizal growth responses of various plants has been shown by various workers to decrease with defoliation or clipping treatment. For instance, Daft and El-Giahmi (1978) showed that differences in size between mycorrhizal and non-mycorrhizal maize and tomato plants decreased with increasing

levels of defoliation. In this study, shoot yields of mycorrhizal white clover are significantly greater than those of the non-mycorrhizal plants prior to the clipping treatment. At the final harvest after clipping, there were no significant differences in the shoot dry weights of mycorrhizal and non-mycorrhizal plants in the control treatments for the pathogens. Significant differences occurred, however, in mycorrhizal and non-mycorrhizal plants treated with *C. fertilis* and *T. basicola*. This suggests that mycorrhizal plant yields are not as severely affected by the pathogens even after clipping treatments.

The production of extensive root rot on clover plants by the three pathogens suggests that these common fungal pathogens could be of importance in grazed pastures. The mechanism responsible for the increase in root rot with defoliation is not known. Various workers (e.g. Lukezic *et al.*, 1969) suggested that defoliation reduces the carbohydrate supply to plant roots and causes a depletion of root carbohydrate reserves. It has been shown by Evans (1973) that defoliation of young white clover plants could depress root elongation to 5% of that of undefoliated plants. Increased root rot and decreased root growth following defoliation would therefore have a combined effect in reducing the effectiveness of the root system, thus affecting shoot yield.

White clover inoculated with macerated mycelia and spore suspensions of the pathogens at various intervals developed a range of root rotting, with seedlings inoculated at transplanting (day 0) showing the most severe symptoms. Apart from infection by *C. fertilis*, where there was a higher percent root infection and reduction in root growth for non-mycorrhizal

plants, the degree of damage caused by *F. avenaceum* and *T. basicola* roots of mycorrhizal and non-mycorrhizal plants did not differ greatly. In all the treatments, however, mycorrhizal plants continued to give significantly greater shoot and root yields.

Disease severity of plants inoculated with the three pathogens at days 28 and 42 appeared to decrease compared with the day 0 treatment. The decrease is particularly pronounced for the mycorrhizal plants, suggesting that prior infection by *G. fasciculatus* confers a greater tolerance on plants to infection. This beneficial effect of the mycorrhizal fungus appears to be negated in plants inoculated at day 56. All the three pathogens caused significant reduction in shoot yields of the mycorrhizal plants. Large shoot yield differences occur between the mycorrhizal and non-mycorrhizal plants. A comparable large reduction in root fresh weight of non-mycorrhizal plants was produced by *C. fertilis* and *F. avenaceum* but reduction in shoot yield occurred only in the *C. fertilis* treatment.

The increase in disease severity in plants inoculated at day 56 may be related to senescence of the roots in these plants. The increase is particularly more marked in the mycorrhizal treatment and is probably related to the greater amount of growth produced compared to the non-mycorrhizal plants, even before inoculation with the pathogen (Tables 5.5, 5.6). The relationship between plant age and susceptibility appears to support the suggestion by Yarwood (1959) that plants at younger and older stages are most susceptible to disease, with greatest resistance at the middle stages of the plant life.

When the pathogens were cultured in oatmeal prior to inoculation, infection was again produced on the roots of both mycorrhizal and non-mycorrhizal plants but disease severity appeared to be decreased compared with using macerated mycelia and spore suspension as the inoculum. Differences in the results of these studies suggest the importance of environmental conditions on root rot development of white clover. Apart from difference in the inoculum media used, the experiment with macerated mycelia and spore suspension was conducted in a growth cabinet with 20-25^{o}C at 16 h daylight. The experiment with ground oatmeal culture was conducted in a glasshouse at 10-30^{o}C. The light intensity in the latter experiment was much higher - the experiment being conducted in the summer. There was evidence of a host resistant mechanism operating in reducing the colonization of the pathogens within the root tissue, especially with *C. fertilis* and *F. avenaceum*. Lignituber-like outgrowths were of common occurrence in roots inoculated with *C. fertilis*, while hyphae of *F. avenaceum* appeared to be confined to groups of cells corresponding to necrotic areas.

C. fertilis appeared to produce the same degree of damage on the roots of mycorrhizal and non-mycorrhizal plants, but no effect on their shoot yields. With *T. basicola*, shoot and root yields of non-mycorrhizal plants were significantly reduced, but not of mycorrhizal plants. *F. avenaceum* did not cause reduction in shoot or root yields of mycorrhizal plants, but a relative reduction in shoot yield of 28% in non-mycorrhizal plants. This suggests that in situations where detrimental effects are produced by the pathogens with respect to plant yield, they are less severe in the mycorrhizal treatment.

Mycorrhizal root infection was significantly reduced only by *C. fertilis* when compared with the distilled water control but not with the autoclaved inoculum control. The significant reduction in mycorrhizal root infection by each of the three pathogens is interesting. The cause for this reduction could be the introduction of nutrient into the soil with the rich inoculum media used for growing the pathogens. However, the reduction in mycorrhizal root infection in each case is not sufficient to lower shoot yields significantly.

CHAPTER 6

CONCLUDING DISCUSSION

C. fertilis, *F. avenaceum* and *T. basicola*, three fungi commonly associated with the roots of white clover were shown to be capable of invading and colonizing roots of plants under the conditions of these studies, producing necrotic lesions or discoloration. The extent of colonization of the host roots and the detrimental effect produced varied according to the environmental conditions. Disease severity was greater for plants inoculated after germination or at a more mature stage and then given foliage-clipping treatment.

The results of interaction studies indicate that the mycorrhizal fungus *G. fasciculatus* does not provide complete protection to infection of roots by the three pathogens. Shoot yield, and in some instances, root yield of mycorrhizal plants, appeared to be less severely affected compared to the non-mycorrhizal plants. The beneficial effect of *G. fasciculatus* is shown, however, to be negated in plants inoculated at a more mature stage, suggesting that the effect of the mycorrhizal fungus varies according to the conditions of the study.

Various possible mechanisms promoting the beneficial effect of VA mycorrhiza are outlined in the General Introduction. Daft and Okusanya (1973) showed that mycorrhizal plants

usually have more vascular bundles and greater lignification of the xylem vessels. Schonbeck (1979) suggested that a more intensive vascular system of the mycorrhizal plants will enable a greater translocation of nutrients, impart greater mechanical strength and diminish detrimental effects resulting from the colonization of the vascular system. Infection of tomato plants with the VA mycorrhizal fungus, *Glomus mosseae*, has been shown to decrease the amount of damage caused by *Fusarium oxysporum* (Dehne & Schonbeck, 1975). In this study, hyphae of both *F. avenaceum* and *T. basicola* could spread to the xylem vessels in severe infection. A more developed vascular system in the mycorrhizal plants could be the cause for a smaller relative reduction in shoot or root yield.

The development of more callosities or lignitubers at the penetration sites has been suggested to cause a greater resistance to penetration of *Pyrenochaeta terrestris* in mycorrhizal onion roots (Becker, 1976). Similar lignituber-like outgrowths were produced in white clover roots in response to infection or attempted penetration by *C.fertilis* and could be an important factor in the beneficial effect of mycorrhizal plants. Experiments to investigate the role of these lignituber-like outgrowths must take into consideration the time of their initiation, the speed of subsequent development as well as their composition since these are important factors that possibly govern the success or failure of lignitubers in preventing the entry of pathogens into host cells.

A mechanism based on changes in the free amino acid levels in the mycorrhizal roots has been proposed by Baltruschat and Schonbeck (1975) to increase plant resistance to infection by *T. basicola*. They suggested that the high content of arginine in mycorrhizal roots strongly inhibited the production of chlamydospores. In the case of white clover, chlamydospores of *T. basicola* were observed in the roots of infected mycorrhizal plants. In some instances, their presence was also noted in the vascular tissues. Differences in observation could have been related to the different inoculum densities of *T. basicola* used in both studies. Baltruschat and Schonbeck (1975) found that inhibition of *T. Basicola* chlamydospore production decreased as the inoculum density increased.

Davies and Mange (1980) found that the beneficial effect of *G. fasciculatus* in soil of low phosphorus was negated by *Phytophthora parasitica* in soil applied with high phosphorus levels. They suggested that tolerance to *P. parasitica* root rot in citrus is caused by the ability of mycorrhizal roots to absorb more phosphorus and possibly other minerals than non-mycorrhizal plants. Experiments conducted using soil applied with perhaps two or three levels of phosphorus would be useful in elucidating the mechanism for the beneficial effect of *G. fasciculatus* on root rot of white clover.

In relation to the above suggestion that the beneficial effect of mycorrhizal roots is due to its ability to absorb more phosphorus than non-mycorrhizal roots, Bowen (1978)

also postulated that growth of mycelial strands and hyphae into soil from mycorrhizal increases 'rooting-intensities' at less energy cost than production of new roots and is a highly effective mode of compensating for root loss by disease. Furthermore, the hyphae are not susceptible to the same diseases as the roots.

Under the conditions of this study, extensive root rotting occurs on inoculation with the pathogens. It is, however, widely recognized that in the field conditions, pathogenicity of many commonly isolated fungi may not be exhibited in vigorously growing plants. Plants in the field usually succumb to root rot gradually, often after they have been exposed to stress conditions (Latch & Skipp, In press). The physical and biological environment of the host plants play an important role in root rot development. Hence, attempts to introduce white clover more widely into infertile hill country and other unfavourable areas, should not overlook the possibility that the increased stresses on the plants imposed by these environments could lead to the development of root rot and persistence problems. As shown from the results of this and other studies, the introduction of a suitable VA mycorrhizal fungus into the field might help prevent the development of root rot or reduce their detrimental effects on plant yields. The outcome of the interaction should perhaps be determined for each host-pathogen mycorrhiza combination, since different VA mycorrhizal fungi have been shown to confer variable tolerance or resistance to

the same pathogen on different hosts (Davis & Menge, 1980).

In the last decade, transmission electron microscopy has been employed by various workers to elucidate details in the life history and other aspects of the mycorrhiza-host associations. In this study, various novel features associated with the intercellular hyphae of *G. fasciculatus* were shown. These include considerably thickened walls, which in some instances lead to the near occlusion of the protoplast.

The intercellular hyphae of most VA mycorrhizal fungi reported have relatively thin walls (e.g. Cox et al., 1975; Holley & Peterson, 1979; Scannerini, et al., 1975). In this study, the thick walls appear to represent secondary walls that develop as the hyphae mature.

Hypotheses are proposed as to the role of these thick-walled hyphae in the life history of the endophyte. One hypothesis is that they may serve as apoplastic pathways for the transport of solution through the hyphae system. To test this system, lanthanum nitrate and uranyl acetate tracers (Fineran & Gilbertson, 1980) could be used in further studies to detect the presence of apoplastic spaces within the thick walls.

Another feature of intercellular hyphae from the older roots is the development of intrahyphal hyphae. Intrahyphal hyphae have not been reported in ultrastructure studies of the coarse endophyte although they have been noted for the fine endophyte, *G. tenuis* (Gianinazzi - Pearson, et al., 1981). The sporadic occurrence of intrahyphal hyphae in intercellular

hyphae located in matured or moribund cortical cells suggests that they are likely to be related to the increasing age of the endophyte and host cells as found also by Chan and Stephen (1967) for their material. They may develop when portions of the hyphal system become damaged or degenerated. Hyphal regrowth from the contiguous unaffected portion of the original hypha would be able to grow within the empty lumen, which probably provides an easier route for the establishment of the hypha than repenetration of the matured tissues of the roots.

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APPENDIX 1

LOW TEMPERATURE STAINING OF VESICULAR-ARBUSCULAR MYCORRHIZAE USING LACTOPHENOL TRYPAN BLUE (ABBOTT, PERS. COMM.)

1. Roots were placed in test tubes and covered with 10% KOH.

2. Test tubes with roots were left for 4h in a water-bath preheated to 60^{o}C.

3. KOH was decanted off and roots rinsed in water.

4. Roots were then rinsed in 10% HCl and drained.

5. They were covered with 0.05% lactophenol trypan blue and returned to water bath for 30-60 min (in fumehood).

6. Test tubes were removed from water bath, covered with Parafilm 'M' and allowed to cool in fumehood.

APPENDIX 2

PREPARATION OF ROOTS FOR OPTICAL MICROSCOPE STUDIES
(A modification of Johansen's method, 1940)

FIXATION

Root segments (0.5 cm) were fixed in 3% glutaraldehyde made up in 0.1 M sodium phospate buffer at pH 6.8.

TERTIARY-BUTYL ALCOHOL DEHYDRATION

Dehydrating solutions were added to roots in specimen jars and changed in the following order:-

Room temperature

50% T.B.A. (1h)

70% T.B.A. (overnight)

85% T.B.A. (1h)

95% T.B.A. (1h)

100% T.B.A. (1h)

40°C oven

Pure T.B.A. (1h)

Pure T.B.A. (1h)

Pure T.B.A. (overnight)

INFILTRATION

50/50 T.B.A. and *'Paraplast' Plus in 40°C oven (1 day)

Paraplast Plus in 60°C oven (1 day)

Paraplast Plus in 60°C vacuum oven (1 day)

Paraplast Plus in 60°C vacuum oven (1 day)

*'Paraplast' Plus tissue embedding medium - a compound of purified paraffin and plastic polymers manufactured by Lancer, Brunswick Co. U.S.A.

EMBEDDING

Root segments were embedded in melted 'Paraplast' Plus in a plaster boat and allowed to cool under water.

APPENDIX 3

SAFRANIN - FAST GREEN STAINING METHOD
(A modification of Jensen's method, 1962)

1. Safranin was prepared by dissolving 1 g of safranin in 100 ml of 60% alcohol and filtered.

2. Fast-green was prepared by dissolving 0.5 g of fast-green in 100 ml of 95% alcohol and filtered.

3. Root sections mounted on slides were passed through the following solutions:-

xylol (wash)
xylol (2 runs of 5 min each)
100% alcohol (1 min)
95% alcohol (1 min)
80% alcohol (1 min)
70% alcohol (1 min)
safranin (4 h)
distilled water (rinse)
60% alcohol (5 sec)
70% alcohol (5 sec)
80% alcohol (5 sec)
95% alcohol (5 sec)
fast green (2 min)
100% alcohol (2 runs of 5 sec each)
xylol (3 runs of 5 min each).

APPENDIX 4

PERIODIC ACID - SCHIFF'S STAINING METHOD

(A modification of McManus's method, 1948)

1. Metabisulphite solution was prepared by dissolving 1 g potassium metabisulphite in 10 ml of 1N HCl and 200 ml of distilled water.
2. Schiff's reagent was prepared as follows:-
   1 g basic fuchsin was added to 200 ml boiling water, allowed to dissolve and cool to 50°C. Potassium metabisulphite (2 g) was added, dissolved and cooled to room temperature. Concentrated HCl (2 ml) was then added and the flask stoppered with a cotton wool bung and left in the dark overnight. Decolourising charcoal was added, the flask shaken and solution filtered. The reagent was kept in a dark box at 4°C until use.
3. Root sections mounted on slides were passed through the following solutions:-

   xylol (2 runs of 5 min each)
   100% alcohol (1 min)
   95% alcohol (1 min)
   80% alcohol (1 min)
   70% alcohol (1 min)
   60% alcohol (1 min)
   50% alcohol (1 min)
   30% alcohol (1 min)
   distilled water (1 min)
   1% aqueous periodic acid (3 min)
   running tap water (10 min)

Schiff's reagent (5 min)

Metabisulphite solution (5 min)

running tap water (till colour appears)

30% alcohol (1 min)

50% alcohol (1 min)

60% alcohol (1 min)

70% alcohol (1 min)

80% alcohol (1 min)

95% alcohol (1 min)

0.5% alcoholic fast green (5 sec)

100% alcohol (3 runs of 5 sec each)

xylol (2 runs of 1 min each and then 1 of 5 min)

APPENDIX 5

WHITE CLOVER SHOOT PHOSPHORUS CONCENTRATION DETERMINATION AND RESULTS

Dried shoot tissues were analysed for phosphorus concentrations, after digesting in $HC\ell O_4/HNO_3$, by the phosphovanadomolybdate method of Kitson and Mellon (1944). As there was insufficient material for analysis in some treatments, shoot tissues from the different replicates were pooled within each treatment. Results of shoot phosphorus concentrations at the different harvests in Tasman and Pawson silt loam were shown in the following tables.

Table (i) Phosphorus concentrations in the shoots of mycorrhizal inoculated (M) and uninoculated (N) plants at harvests 1 and 2. (Tasman silt loam).

Applied P (mg/pot)	Shoot P concentration (%)			
	Harvest 1		Harvest 2	
	M	N	M	N
0.0	0.15	0.14	0.13	0.11
10.7	0.17	0.16	0.15	0.13
37.5	0.22	0.21	0.19	0.17
131.3	0.25	0.26	0.26	0.21
459.6	0.44	0.41	0.33	0.34

Table (ii) Phosphorus concentration in shoots of mycorrhizal inoculated (M) and uninoculated (N) white clover at harvests 1, 2 & 3 (Pawson Hill silt loam).

Applied P (mg/pot)	Shoot phosphorus concentration (%)					
	Harvest 1		Harvest 2		Harvest 3	
	M	N	M	N	M	N
0.0	0.22	0.13	0.12	0.09	0.15	0.11
9.2	0.25	0.16	0.11	0.11	0.15	0.12
27.6	0.27	0.26	0.15	0.15	0.15	0.17
82.8	0.39	0.35	0.21	0.21	0.22	0.26
248.4	0.50	0.64	0.27	0.33	0.32	0.40

APPENDIX 6

FUNGAL GROWTH MEDIA

Potato Dextrose Agar (PDA)

200 g potato
115 g dextrose
20 g agar
1 litre water

The potatoes were peeled and diced, boiled for an hour and the mixture passed through a fine sieve. Agar was added to the mixture and boiled until dissolved. Dextrose was added and the mixture stirred. They were then autoclaved in 250 ml Erlenmeyer flasks at 121°C for 20 min, after adjusting pH to 5.2 with dil. HCl.

Acidified PDA for reisolation studies (Tuite, 1969)

Lactic acid (25%) was added to the above medium (3-5 drops per 100 ml), after autoclaving and before pouring into plates.

V_8 Juice Agar (V_8A)

200 ml V_8 juice
$CaCO_3$ (added till pH = 5.4)
20 g agar
800 ml distilled water

The V_8 juice, agar and distilled water were mixed well and brought to the boil. $CaCO_3$ was added until pH = 5.4. The mixture was then placed in 250 ml Erlenmeyer flasks and autoclaved at 121°C for 20 min.

Potato Sucrose Agar (PSA)

500 ml potato extract*
20 g sucrose
20 g agar
500 ml distilled water

The potato extract and water were mixed together and the sucrose and agar added. The mixture was heated slowly until the agar was dissolved and the pH adjusted if necessary to 6.5 with calcium carbonate. It was then dispensed in 250 ml Erlenmeyer flasks and autoclaved at 121°C for 20 min.

* potato extract was prepared from 1800 g of potato, peeled, diced and suspended in muslin in 4500 ml of water and boiled for 10 min. The potatoes were then discarded and the extract placed in one litre Erlenmeyer flasks and autoclaved at 121°C for 20 min. It was then stored in a refrigerator for use as required.

APPENDIX 7

PREPARATION OF LACTOPHENOL COTTON BLUE

Lactophenol		Cotton blue solution	
lactic acid	30 ml	saturated aniline blue	
phenol	30 g	solution	10 ml
glycerol	30 ml	(1 g in 10 ml water)	
distilled water	30 ml	glycerol	10 ml
		water	80 ml

Lactophenol was prepared by first dissolving phenol in water, lactic acid and finally glycerol was then added.

Cotton blue solution was prepared by adding glycerol and water to the saturated aniline blue solution.

Equal parts of lactophenol and cotton blue solutions were then mixed together.